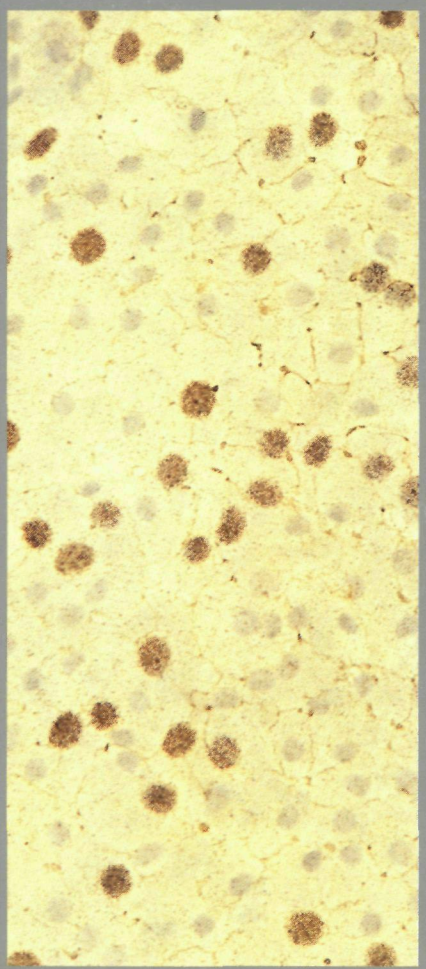
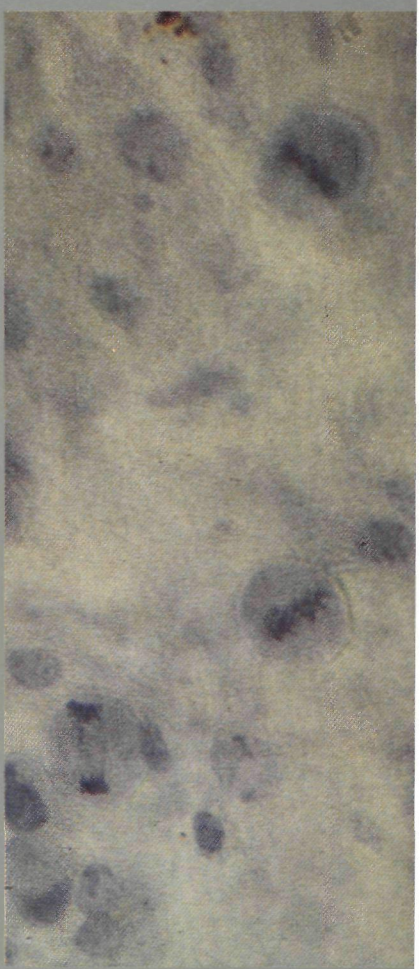
CORNEAL ENDOTHELIAL WOUND HEALING





W.F. TREFFERS

PROMOTORES:

PROF DR. A F. DEUTMAN

PROF. DR C.R. JERUSALEM

CORNEAL ENDOTHELIAL WOUND HEALING

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN

DOCTOR IN DE GENEESKUNDE

AAN DE KATHOLIEKE UNIVERSITEIT VAN NIJMEGEN

OP GEZAG VAN DE RECTOR MAGNIFICUS PROF DR J H G I GIESBERS

VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN

IN HET OPENBAAR TE VERDEDIGEN

OP DONDERDAG 16 SEPTEMBER 1982

DES MIDDAGS TE 4 00 UUR

DOOR

WILLEM FRITS TREFFERS

GEBOREN TE DE BILT

JANSSEN|PRINT NIJMEGEN 1982

The research in Miami was financed in part by grant EY 00031 (D R Anderson, M D) and grant EY 00162 (P Palmberg, M D Ph D), both from Public Health Research Grants by the National Eye Institute, National Institutes of Health, Bethesda, Maryland, USA
An International Research Scholar grant was received from Research to Prevent Blindness, Inc , New York, New York, USA
Financial support was provided by the 'Research Fonds Oogheelkunde' of the University of Nijmegen

Upon completion of this thesis I should like to address a few words of thanks to those who have contributed to its conception and publication.
The research presented in this thesis was done in two worlds; since it started in the Old World, I should like first to thank those who helped me there.
I thank Messrs T.H.M. Arts and P.G.H. Philipsen of the operative department of the central animal laboratory (head: Dr W.J.I. van der Gulden) for their help in performing the animal experiments. And Messrs H.J. Winkens of the Ophthalmological Laboratory (head: Dr R.M. Broekhuyse) and P. van Wichen of the Biological Laboratory of the ENT Department (head: Dr W. Kuijpers) for their help in the preparation of the specimens and the performance of autoradiography.
I am indebted to Mr H. de Boer of the Department of Submicroscopic Morphology (head: Prof. Dr A.M. Stadhouders) for the excellent way in which he performed the scanning electron microscopy for my study.
In the New World my first debt of gratitude is to the Florida Lions Eye Bank (Med. director: V.T. Curtin, M.D.). Without their help I would not have had sufficient human material for the in-vitro experiments. For these experiments I received valuable advice from the tissue culture specialist A.S. Hajek, Ph.D., while E.B. Davis performed all the autoradiography and helped me in processing the specimens.
I thank the photographers F.G. Gonzales and B.A. French for their assistance in obtaining and processing the vast photographic material.
My special thanks go to Jean-Marie Parel, Ing.ETS-G, for the invaluable help he gave me during the year in Miami.
Most of all I thank E.W.D. Norton, M.D., Professor and Chairman, for his help in setting up the in-vivo experiments; without his help and support this study could not have been performed in this form.
For the preparation of the final manuscript I am indebted to Mr A.L. Aan de Kerk for the excellent finish he gave the photographic material, and to Mrs M.L.A. Ackermans - De Leeuw of the medical drafting office, who prepared the drawings.
The manuscript was typed by Mrs C.P. Aan de Kerk - Beekhuizen, and translated into English by Mr Th. van Winsen.

CHAPTER 1 INTRODUCTION 11

CHAPTER 2 CORNEA 13
2.1. Embryology 13
2.2 Anatomy 16
2.2.1 Tear film 16
2.2.2 Epithelium 16
2.2.3 Basement membrane 16
2.2.4 Bowman's layer 17
2.2.5 Stroma 17
2.2.6 Descemet's membrane 18
2.2.7 Cell junctions 19
2.2.7.1 Desmosomes 19
2.2.7.2 Tight junctions 21
2.2.7.3 Gap junctions 21
2.2.8 Endothelium 21
2.3 Physiology 24
2.4 Wound healing 26
2.4.1 Cell cycle 26
2.4.2 General introduction to wound healing 30
2.4.3 Corneal wound healing 31
2.4.3.1 Endothelium 31
2.4.3.2 Stroma 34
2.4.3.3 Combined endothelial and stromal wound 34

CHAPTER 3 MATERIAL AND METHODS 37
3.1 Material 37
3.1.1 In-vivo corneal endothelial wound healing in non-human primates . 37
3.1.1.1 Endothelial loss and wound healing after corneal allograft .. 38
3.1.1.2 Endothelial wound healing 39
3.1.1.3 Endothelial wound healing after corneal autograft 40
3.1.2 In-vitro human corneal endothelial wound healing 41
3.1.2.1 Standardization of endothelial wounds 43
3.1.2.2 Controls 43
3.1.2.3 DNA synthesis? 43
3.1.2.4 DNA synthesis and/or mitosis 44
3.1.2.5 Autoradiographically positive mitotic figure 44
3.1.2.6 Qualitative difference in wound healing between a mechanical and a cryo-induced corneal endothelial lesion 44

3.1.2.7 Influence of exogenous factors 44
3.1.2.8 Scanning electron microscopy of wound healing 45
3.1.2.9 Cross-sections 45
3.1.3 In-vivo human corneal endothelial wound healing 46
3.2 Methods 46
3.2.1 Organ culture 47
3.2.2 Trypan blue (vital) staining 47
3.2.3 Silver staining 47
3.2.4 Preparation of a flat corneal endothelial specimen 47
3.2.5 Autoradiography 48
3.2.6 Nuclear staining 50
3.2.7 Scanning electron microscopy 51
3.2.8 Transmission electron microscopy 51

CHAPTER 4 RESULTS 52
4.1 In-vivo corneal endothelial wound healing in non-human primates 52
4.1.1 Endothelial loss and wound healing after corneal allograft .. 53
4.1.2 Endothelial wound healing 60
4.1.3 Endothelial wound healing after corneal autograft 64
4.2 In-vitro human corneal endothelial wound healing 69
4.2.1 Standardization of endothelial wounds 69
4.2.2 Control 71
4.2.3 DNA synthesis? 73
4.2.4 DNA synthesis and/or mitosis 77
4.2.5 Autoradiographically positive mitotic figure 82
4.2.6 Qualitative difference in wound healing between a mechanical and a cryo-induced corneal endothelial lesion 87
4.2.7 Influence of exogenous factors 88
4.2.8 Scanning electron microscopy of endothelial wound healing . 89
4.2.9 Cross-sections 94
4.2.9.1 Normal endothelium 94
4.2.9.2 Normal endothelium at silver staining 97
4.2.9.3 Wound healing at silver staining 100
4.2.9.4 Wound healing at silver staining and autoradiography 100
4.3 In-vivo human corneal endothelial wound healing 102

CHAPTER 5 CONCLUSIONS AND DISCUSSION 108

CHAPTER 6 SUMMARY 116

SAMENVATTING 119

LITERATURE 122

INTRODUCTION

Lesions of the endothelial lining of the cornea, which is only one layer of cells thick, occur after virtually every ophthalmological operation, and especially after intraocular procedures. Sooner or later they must lead to functional decompensation of the corneal endothelium. During life this endothelial cell loss results in a number of cases in loss of corneal clearness.

In such cases function can be restored only by a penetrating corneal graft. Whether the graft remains clear, and visual acuity intact, partly depends on the number of endothelial cells left on the graft after the operation, and their ultimate function. Depending in part on the original pathogenesis, the postoperative course of a corneal graft is not infrequently complicated in its turn by functional decompensation of the transplanted donor endothelium. Several factors can play a role in the pathogenesis of opacification of a corneal graft. Differentiation between immunological factors and disturbances of wound healing is often difficult.

Although much research has been and is still being done with regard to the role of immunology in corneal grafts, a laboratory test which either demonstrates or excludes an allograft reaction is not yet available. Very often, therefore, it is on the basis of clinical experience that a conclusion is reached.

Far less research has focused on wound healing and the regenerative power of human corneal endothelium. And no research has been devoted to the endothelial lesions which occur as a direct result of the surgical trauma caused by a corneal graft. Yet these non-immunological factors are of importance for the ultimate result of a corneal graft.

Histopathological examination of decompensated corneal grafts, regardless of whether the decompensation is clinically diagnosed as immunological or not, nearly always reveals a disturbance in wound healing in the deeper part of the wound.

The generally accepted theory is that, in primates, corneal endothelial lesions heal solely by migration of the remaining vital cells, without cell division. The endothelial cells involved in the healing process become flatter, and their individual surface area increases. The normal, regular hexagonal pattern of the endothelial cells is lost in this process, to be replaced by an irregular pattern. Monkeys are regarded as the most suitable test animals, in which corneal graft features reflect those of human corneal grafts as faithfully as possible. The monkey corneal endothelium, unlike that in the often used young adult rabbit, is thought to be incapable of cell division as part of endothelial wound healing. In the young adult rabbit it has been clearly demonstrated that corneal

endothelial wound healing involves both migration and proliferation of cells. This thesis discusses the influence of non-immunological factors on wound healing, with emphasis on wound healing in the deeper layers of a corneal wound. Primarily a study was made of the localization and extent of corneal endothelial lesions resulting from the surgical trauma of a corneal graft. This study was performed on adult monkeys and also encompassed a study of the pattern of wound healing on the inner surface of the cornea.

The results prompted a further study of aspects of the corneal endothelial wound healing pattern, with special emphasis on the question whether or not the endothelium is capable of cell division as part of the healing proces. The results obtained in this study prompted an in-vitro an in-vivo study of human corneal endothelial wound healing.

For this study it was necessary to develop a new technique of investigation which makes it possible to study both migration and proliferation in the same specimen. This method would have to be applicable, moreover, to both purely endothelial wounds and combined endothelial and stromal wounds.

CHAPTER 2

CORNEA

2.1 EMBRYOLOGY

Once the neural crest has formed, the proximal neuroectoderm which is to form the optic cup develops from the primitive nucleus. This tissue later becomes the neuroectodermal part of the eye. As soon as the out-pouching optic cup has approached the ectoderm, it induces the latter to form the lens placode (Duke Elder and Cook 1963). In this thickened ectodermal area - the lens placode - a depression forms which in a later stage of development becomes the ectodermal invagination.

This invagination is separated from the ectoderm by in-growth of mesenchymal tissue. The invagination (now called lens vesicle), surrounded by some mesenchymal tissue, disappears almost entirely in the already invaginated optic cup, which is of neuroectodermal origin. The formation of the lens vesicle is completed in the fifth week of embryonic development: the 9-10 mm stage (Ewer 1970). The development of the anterior segment of the human eye closely resembles that in the non-human primate (Wulle and Lerche 1969; Ozanics et al. 1977). Ozanics et al. (1977) studied the development of the cornea in the non-human primate *Macaca mulatta*. They found that, as soon as the lens vesicle has separated from the ectoderm on the 36th day and lies in the optic cup, a narrow space forms between the lens and the ectoderm above it. This narrow space around the lens vesicle is filled with a vitreous-like substance which contains fibrils with an average diameter of 20.8-29 nm. Anterior to the lens, between the lens and the ectoderm, a basement membranelike substance is localized which may be of ectodermal origin (Ozanics et al. 1977).

A vascular membrane posterior to the lens (Düblin 1970) originates from the hyaloid artery system. The distance between this capsule and the posterior lens surface equals that between the ectoderm and the anterior lens surface (Mann 1950). The space on the anterior side (anterior vitreous) contains a few fibrous adhesions between lens and ectoderm. Similar adhesions are found between the vascular membrane and the posterior lens surface.

On the 40th day of development of the non-human primate embryo, mesenchymal cells localized at the margin of the neuroectodermal optic cup grow in anterior direction between lens and ectoderm (Ozanics et al. 1977). Mann (1950) described a layer separating the ectoderm from the anterior vitreous as a condensation membrane which serves to guide the in-growing mesenchyma. This primary in-growth (Düblin 1970) has also been described as first wave of

mesenchymal in-growth The primary mesenchymal layer differentiates subsequently to become the endothelium Studies of the origin of ocular and periocular tissue by means of heterotopic grafts revealed that the corneal endothelium in chick embryos is of neuroectodermal origin (Johnston et al 1979) Experiments in which the lens of a chick embryo was removed, demonstrated that primary mesenchymal in-growth did not take place in these circumstances (Zinn 1970) and that, consequently, neither endothelium nor Descemet's membrane was formed

The presence of the lens is a prerequisite for undisturbed development of the anterior segment (Coulombre 1969) In the stage of primary mesenchymal in-growth (the 17-18 mm stage of human embryonic development, 6 weeks) the space between ectoderm and primary mesenchymal layer is still devoid of cells (Dublin 1970, Ewer 1970) Completion of primary mesenchymal in-growth is followed by the second wave of mesenchymal in-growth It has not been established with certainty whether this in-growth takes place in one phase (Dublin 1970, Ewer 1970) or in two phases (Mann 1950, Duke Elder and Cook 1963, Ozanics et al 1977)

The single-phase hypothesis postulates that mesenchymal proliferation occurs simultaneously on either side of the primary mesenchymal layer, and that the part on the ectodermal side becomes the corneal stroma, while that on the lens side becomes the primary pupillary membrane This primary pupillary membrane becomes vascularized secondarily and forms the stroma of the iris (Dublin 1970)

The two-phase hypothesis postulates that primary mesenchymal in-growth is followed by a second wave of mesenchymal in-growth, which is confined to the space between the primary mesenchyma and the ectoderm and subsequently forms the corneal stroma The primordium of the corneal stroma having thus been established, a third wave of mesenchymal in-growth takes place from the margin of the optic cup This tissue forms the primary pupillary membrane, which is vascularized secondarily to form the stroma of the iris The primordium of the stroma of the iris (vascularization of the primary pupillary membrane) is established in the 30-40 mm stage (9-10 weeks), and the central part subsequently regresses (Pearson 1975) to form the pupil as it does in the single-phase hypothesis

In experiments reported by Zinn (1970), primary mesenchymal in-growth failed to occur after lens extraction but later mesenchymal in-growth did occur, either in one or in two phases These experiments showed that, instead of normal corneal stroma, a kind of sclera-like tissue developed

After primary mesenchymal in-growth cell junctions begin to form on the apical side of the cells, i e the side toward the lens (Wulle and Lerche 1969, Waring et al 1974) These cell junctions are initially local but gradually come to encompass the entire apical circumference of the cell (Wulle et al 1974) At the same time, gap junctions form between the lateral cell boundaries (see

subsection 2.2.7). This process starts in the 7th-8th week and is completed during the fourth and fifth months of gestation. This coincides with the start of aqueous humour production (Wulle et al. 1974).

The cells of primary mesenchymal in-growth (now named endothelium) synthesize the endothelial basement membrane: Descemet's membrane. This membrane becomes distinguishable from the third (Wulle and Lerche 1969) or from the fourth month of gestation on (Mann 1950; Waring et al. 1974), dependent on the method of investigation used. At birth, Descemet's membrane has a thickness of 3 μ (Waring et al. 1974).

Phylogenetically, the corneal stroma can be divided into two layers which, together, represent the various layers of the skin (Walls 1942; Fine and Yanoff 1979). The mesenchyma localized more immediately below the ectoderm forms the anterior one-third of the corneal stroma and the subconjunctival tissue,

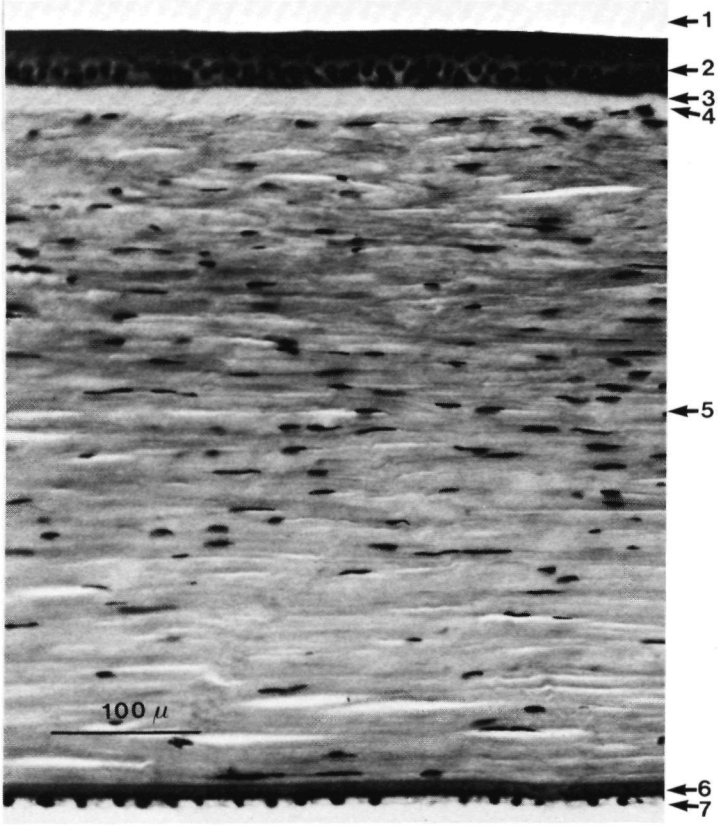


FIG. 1 *Light microscopy of a normal human cornea (HE stain). 1. Tear film, lost during histological processing. 2. Epithelium. 3. Basement membrane. 4. Bowman's layer. 5. Stroma. 6. Descemet's membrane. 7. Endothelium.*

while the deeper mesenchyma forms the posterior two-thirds of the corneal stroma. These two layers are distinguishable as such in lower animal species (fishes and some amphibians) but not in man (Walls 1942). In man, lightmicroscopic examination does reveal a difference in fibrillar arrangement (Fine and Yanoff 1979) which corresponds with these two layers.

2.2 ANATOMY

The cornea is an avascular tissue localized on the anterior aspect of the eyeball. The cornea is normally quite clear and transparent. It consists of six layers (McTigue 1967; Setälä 1980), supplemented by a seventh layer: the tear film (McTigue 1967) (fig. 1).

2.2.1 Tear film

This seventh layer is lost in histological processing and not found in prepared specimens (McTigue 1967). The tear film can be observed, however, with the slit-lamp. The tear film plays an important role in keeping the epithelium moist. Strictly speaking, however, it is not an anatomical part of the cornea (Fine and Yanoff 1979).

2.2.2 Epithelium

The epithelium consists of five or six layers of cells at the centre and of about ten layers of cells at the periphery of the cornea. The epithelium represents about 10-12% of the total thickness of the cornea. The basal layer is also known as germinative cell layer. It is here that new epithelial cells are formed. After each cell division one of the two daughter cells remains behind to serve as new germinative source for the next cell division, while the other daughter cell migrates upwards. It takes about 3-7 days before a newly formed epithelial cells is shed.
The epithelial cells are linked together by means of desmosomes (Jakus 1961; Farquhar and Palade 1963); and the basal cells are linked to the underlying basement membrane by hemidesmosomes (Waltman and Kaufman 1970; Fine and Yanoff 1979) (see subsection 2.2.7).

2.2.3 Basement membrane

The basement membrane is a separate part of the cornea, localized between the

epithelial cells and Bowman's layer. The membrane is produced prenatally by the epithelium. Cultures of embryonic chick corneal epithelium are capable of synthesizing such a basement membrane (Hay and Dodson 1973). The basement membrane of the cornea is entirely comparable with that in other tissues (Maurice 1957). Its thickness is 60-65 nm (Waltmann and Kaufman 1970). Bowman's membrane as seen at light microscopy, can be subdivided into two separate entities: the basement membrane and Bowman's layer (Jakus 1964).

2.2.4 Bowman's layer

This separate corneal layer (McTigue 1967; Fine and Yanoff 1979) has a thickness of 10-16 μ. The acellular layer is localized immediately below the basement membrane and is a specialized layer of the corneal stroma. The primordium of the collagenous fibres which constitute Bowman's layer, is established prenatally. When this layer is damaged later in the course of life, the same prenatal collagen can no longer be synthesized. The defect is filled with epithelial cells or cicatricial tissue (Fine and Yanoff 1979).
The collagenous fibres of Bowman's layer show a random arrangement. Apart from a minor difference in diameter, the fibres resemble the stromal fibres. Bowman's layer is traversed by delicate canaliculi which also traverse the basement membrane. These canaluculi accommodate the corneal nerves, which terminate in free endings among the epithelial cells (Fine and Yanoff 1979; Binder et al. 1980).

2.2.5 Stroma

This layer accounts for about 90% of the total thickness of the cornea, which ranges from 0.5 mm at the centre tot 0.9 mm at the periphery. Corneal thickness shows interindividual differences.
The stroma is made up of collagenous fibrils. These fibrils or fibres are about 19 nm thick on the side of Bowman's layer, the range being 16-24 nm (Jakus 1961). Fibre thickness increases as the depth of their localization in the stroma increases. The fibres have a diameter of 34 nm (range 27-40 nm) on the side of Descemet's membrane (Jakus 1961).
The individual collagenous fibres are so arranged that a number of them together form a band or lamella. Within such a band the fibres are very closely linked. The links to adjacent bands are far less close. Each band extends from one side of the cornea to the other, thus ensuring a very regular stratified corneal structure. The band pattern is very regular in the deeper two-thirds of the cornea, but less regular in the anterior one-third. The anterior lamellae

gradually fuse with Bowman's layer, while the deepest stromal lamellae fuse with Descemet's membrane.
Between the lamellae of the stroma lie the stromal cells, the keratocytes, which present the same cellular appearance as fibrocytes. The keratocytes are spindle-shaped and have long cell processes with which they maintain intercellular contact without forming a true syncytium (Loewenstein and Penn 1967). In transverse sections of the stroma the nuclei of the keratocytes are readily discernible as fairly long structures.
The collagenous fibres of the stroma are embedded in a ground substance which consists of several acid mucopolysaccharides (glucosamine glycans) (Waring et al. 1974; Fine and Yanoff 1979; Waltmann 1981).
The sensory nerves of the cornea enter the cornea in a radial pattern, while they are often still myelinated. They usually lose their myelin sheath a few millimetres from the limbus (Binder et al. 1980).

2.2.6 Descemet's membrane

This corneal layer serves as basement membrane of the endothelium. Descemet's membrane ends at an annular corneal thickening which is known as the line of Schwalbe. A trabecular area is localized posterior to this line. The ciliary muscle inserts on the line of Schwalbe via the scleral spur (Fine and Yanoff 1979; Waltman 1981).
Descemet's membrane is about 3 μ thick at birth (Waring et al. 1974) and increases in thickness in the course of life (Hogan et al. 1971; Leuenberger et al. 1973; Fine and Yanoff 1979). This increase in thickness results from constant deposition of new Descemet material by the endothelium.
Electron microscopy makes it possible to distinguish between an embryonic and a postnatal part of Descemet's membrane. The postnatal part presents a more amorphous, granular appearance (Jakus 1961, 1964; Waring et al. 1974; Fine and Yanoff 1979) (see fig. 50).
The fibrils of Descemet's membrane are embedded in a ground substance identical to the one in which the stromal fibrils are embedded (Waring et al. 1974; Fine and Yanoff 1979; Waltman 1981). The fibrils have a diameter of about 10 nm (Marshall and Grindle 1978). This structure makes Descemet's membrane very homogeneous and elastic. The membrane is highly resistant to invasion of bacteria and leucocytes (Inomata et al. 1970; Waring et al. 1974; Waltman 1981) and also resists the influences of various enzymes such as collagenases and trypsin. Even blood vessels which enter the cornea under pathological conditions, are unable to penetrate this tough membrane (Waring et al. 1974). Descemet's membrane becomes thinner towards its periphery.
The endothelium can sometimes synthesize Descemet material which has prenatal characteristics. This material is deposited locally and irregularly.

When this happens at the periphery of Descemet's membrane, it is regarded as evidence of a physiological process of senescence. These deposits are called Hassell-Henle warts (Fine and Yanoff 1979). Similar bio-microscopic deposits at the centre of Descemet's membrane are regarded as evidence of a pathological process (cornea guttata). Whether this phenomenon involves changed material synthesized by normal endothelium or synthesis of material by a transformed endothelium is unknown (Waring et al. 1974; Fine and Yanoff 1979; Grayson 1979).

2.2.7 Cell junctions

Before discussing the anatomy of the endothelium and its cell junctions, a brief survey of cell junctions is presented. For this purpose the following literature was studied: Muir and Peters 1962; Farquhar and Palade 1963; Overton 1974; Loewenstein 1975; Pappas 1975; Ottersen and Vegge 1977; Fine and Yanoff 1979; Ham and Cormack 1979; Karp 1979.
Cells in suspension show randomly arranged processes: filopodia. The cells seek to establish contact with each other via these filopodia, and start to form cell junctions. As the cells come closer together, their filopodia become shorter; this is followed by interdigitation of the cell membranes of adjacent cells. At some sites the cells are interconnected. These cell junctions have been studied mainly in epithelial cells but also occur among other cell types. The cell junctions found in vertebrates are subdivided into three main groups:
Desmosomes (adherent junctions).
Tight junctions (occluded junctions).
Gap junctions (nexus).

2.2.7.1 Desmosomes

The function of desmosomes is to ensure strong adherence between cells exposed to external mechanical forces, and to distribute these forces. The desmosomes are subdivided into three subgroups: belt desmosomes (zonulae adherentes), desmosomes (maculae adherentes) and hemidesmosomes.
The tonofibrils of a cell form what may be described as an internal cell skeleton. These tonofibrils are attached to the cell membrane, and this attachment has a certain cytological structure (fig. 2).
The term desmosome applies when the adjacent cell has a comparable structure. The term belt desmosome applies when the attachment of the tonofibrils does not end locally but takes a circular course. The term hemidesmosome applies when the cell possesses the cytological features of a desmosome but is bounded, not by another cell but by a basement membrane.

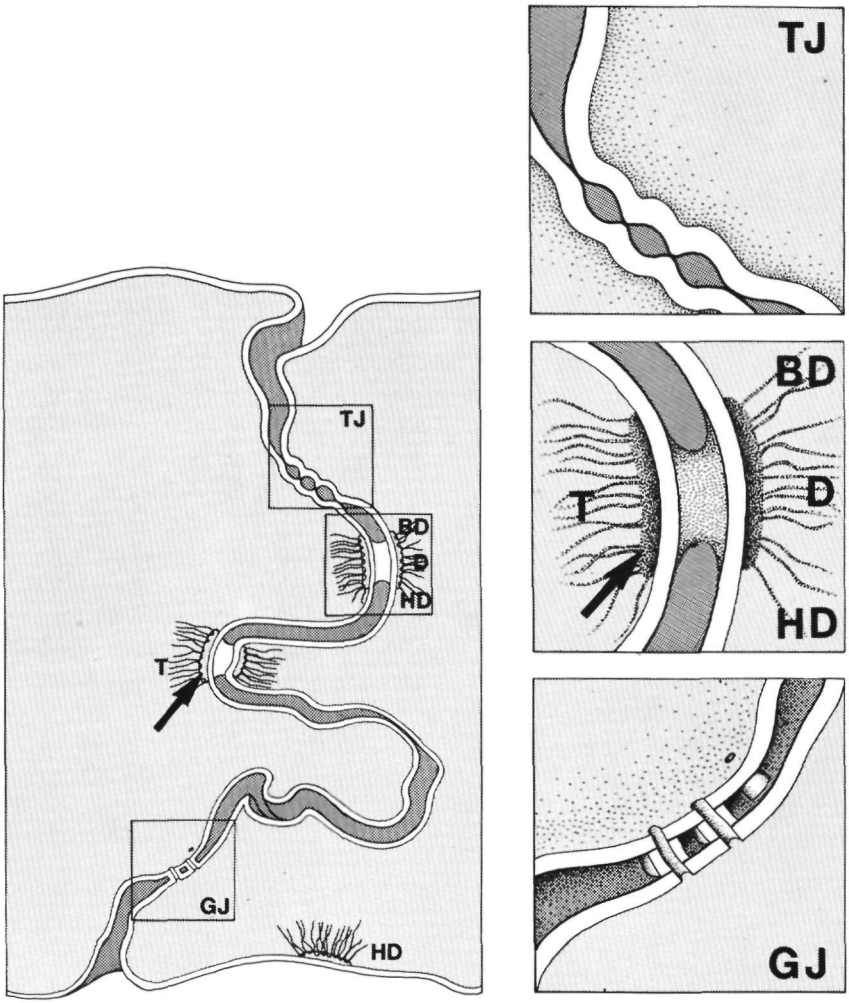


FIG. 2 *Schematic representation of various cell junction types. Desmosome (D), belt desmosome (BD), hemidesmosome (HD), tight junction (TJ), gap junction (GJ).*
Where the tonofibrils (T) attach to the cell membrane, more intensive cytoplasmic densification is observed (arrow). On the right, the junctions are shown at high magnification. The double line represents a cell membrane.

The intercellular space in the case of a desmosomal junction measures 20-35 nm and contains fine filamentous material, which is assumed to serve as intercellular cement. Desmosomal cell junctions are found especially in cells exposed to some considerable force, e.g. in the skin or the cervix uteri.

2.2.7.2 Tight junctions

The function of tight junctions is to close intercellular spaces of cavities, e.g. vascular endothelium and vascular lumen, bladder wall epithelium and bladder cavity. This junction prevents exchanges of ions and small molecules between lumen and intercellular space.
Dependent on the degree of necessity of effective separation between lumen and intercellular space, fewer or more circular, joined rows of cell junctions are formed. In the epithelium of the bladder wall as many as twelve rows may be found - far more than in the vascular endothelium, where less effective separation suffices and where three rows of tight junctions are often found. The cell membranes of the cells with such junctions are fused, and an intercellular space is therefore no longer found, even though the cytoplasm of one cell remains separated from that of the next (fig. 2).
In a lining with a thickness of one layer of cells, these types of cell junction are localized on the apical side. In light microscopy this functional entity is known as terminal bar.

2.2.7.3 Gap junctions

Gap junctions are junctions between adjacent cells whose cell membranes lie close together (2-4 nm) but do not fuse. The gap is bridged by what may be described as canaliculi with an internal diameter of 1.5 nm (fig. 2). Exchange between the cytoplasms of the cells involved takes place through these canaliculi. This exchange can be of a chemical or of an electrical nature.

2.2.8 Endothelium

The endothelium is a lining with a thickness of one layer of cells. Linings of serous spaces are generally called mesothelium, while those of blood vessels and lymphatics are known as endothelium. Both are of mesodermal origin. Some authors describe cells lying adjacent to cavities as squamous epithelium (Bloom and Fawcett 1975). Although the proper term for the inner lining of the cornea is in fact mesothelium, the term endothelium is so widely used in the literature that it will be adopted in this study. Meier (1977) regards this cell layer as posterior corneal epithelium.
At birth, the number of endothelial cells per cornea is about 300,000-500,000 (Svedbergh and Bill 1972; Bourne and Kaufman 1976; Binder et al. 1980). The inner surface area of the cornea measures about 100 mm^2, and the cellular density therefore varies from 3000 to 5000/mm^2. The cells are about 4-5 μ thick and have a diameter of 15-30 μ (Binder et al. 1980). In young individuals

the nucleus is located centrally in the cell. The apical cell boundary shows a regular hexogonal pattern. With increasing age, the nuclei assume a more eccentric position in the cells. The apical cell boundary loses its regular pattern, and marked interindividual variations develop (fig. 3). This is the result from a decrease in the number of endothelial cells per unit of surface area, as clearly demonstrated by in-vivo photography of the endothelial cells (= specular microscopy) (Kaufman et al. 1966; Laing et al. 1976).

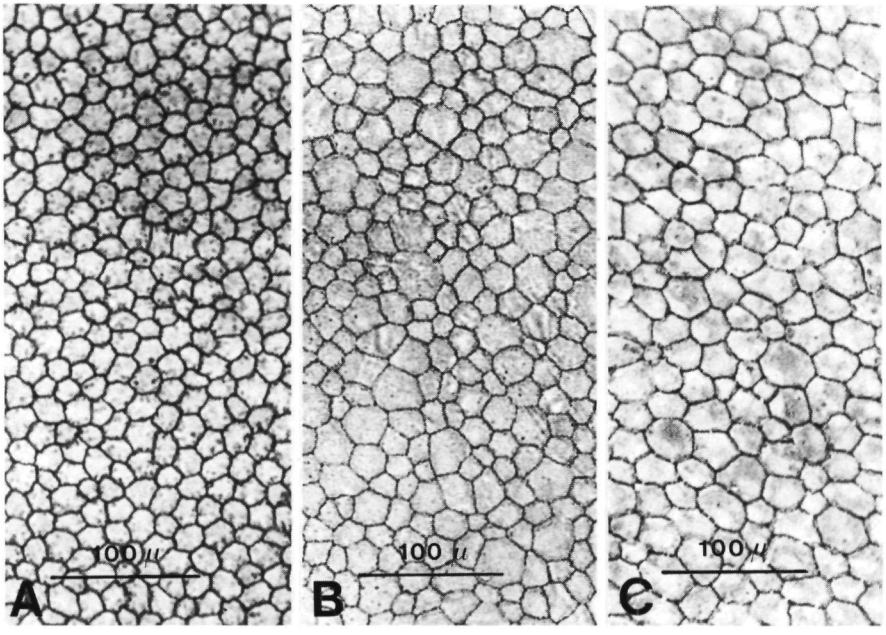


FIG. 3 *Flat preparations of human corneas, silver-stained. A. Donor aged 21. B. Donor aged 45. C. Donor aged 80. Compare individual endothelial cell sizes.*

The cell pattern abruptly changes at the line of Schwalbe (fig. 4). The tissue posterior to the line of Schwalbe is known as trabecular zone. This zone has a sponge-like collagenous structure, in which the spaces are lined with endothelial cells. These spaces end at Schlemm's canal. This zone plays an important role in the drainage of aqueous humour.

The cytostructural features warrant the assumption of an active mechanism of transcellular ion transport. This is suggested by the number of mitochondria which are unusually long (Hogan et al. 1971; Tripathi 1972; Fine and Yanoff 1979; Binder et al. 1980; Setälä 1980). The presence of an extensive endoplasmic reticulum, moreover, indicates protein synthesis. This synthesis is required for the constant synthesis of new Descemet material (Hogan et al. 1971).

The cell junctions of and between the corneal endothelial cells show some

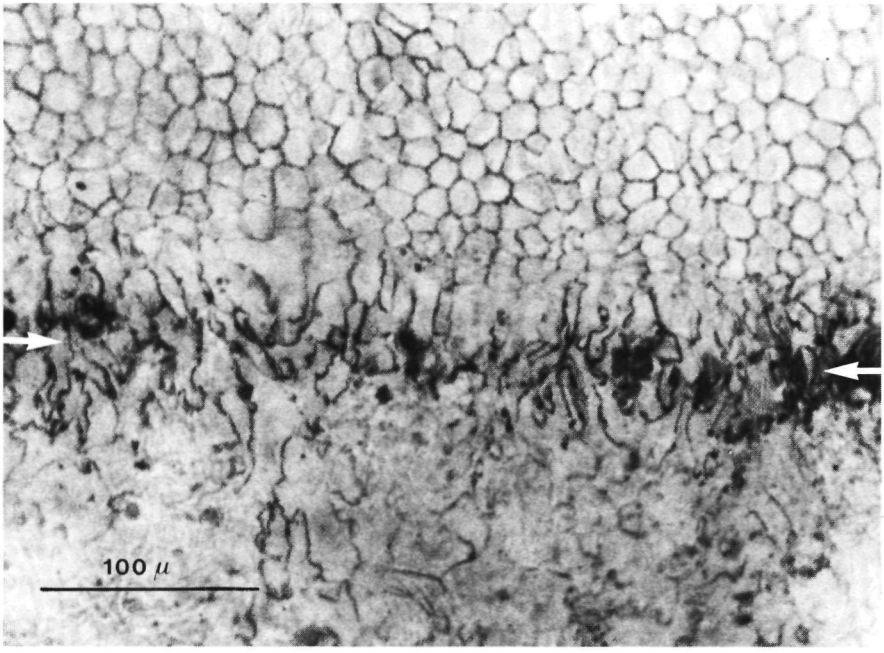


FIG. 4 *Flat preparation of a human cornea. Transition from endothelium (top) via the line of Schwalbe (arrow) to the trabecular zone (bottom). Donor aged 13.*

differences from those described in subsection 2.2.7. The endothelial cells are not attached to Descemet's membrane by hemidesmosomés. The junction between the endothelial cells and Descemet's membrane is established by the constant production of new Descemet membrane fibres by the endothelium. In the absence of hemidesmosomes, the endothelial cells are readily detached from their substratum. This will be the case in particular during intraocular interventions (Pearce et al. 1969; Waring et al. 1974). The intercellular space between endothelial cells measures about 20 nm (Fine and Yanoff 1979). The lateral cell boundaries show very intensive interdigitation although no desmosomes (maculae adherentes) are found (Jakus 1961; Kuwabara 1978; Fine and Yanoff 1979; Binder et al. 1980). On the other hand, Iwamoto and Smelser (1965) reported the presence of an occasional desmosome.
Gap junctions have been demonstrated between the corneal endothelial cells (Iwamoto and Smelser 1965; Ottersen and Vegge 1977; Kuwabara 1978).
On the apical side of the intercellular space between the endothelial cells, two types of cell junction have been demonstrated which separate the intercullular space from the anterior chamber of the eye: a belt desmosome (Iwamoto and Smelser 1965; Tripathi 1972; Kreutziger 1976; Kuwabara 1978; Fine and Yanoff 1979; Binder et al. 1980), and a tight junction (McTigue 1967; Hirsch et

al. 1977; Ottersen and Vegge 1977). This functional entity was known as terminal bar in light microscopy. Electron-microscopic examination of the terminal bar has revealed that it encompasses a belt desmosome and a tight junction, with the latter on the side of the lumen (fig. 2). At high magnification the endothelial cells can be observed to overlap slightly on the side of the lumen. This overlap is known as apical fold (see fig. 47 and 51).
The cell pattern on the luminal side is hexagonal (figs. 3 and 47). This hexagonal pattern is found both in man and in various animal species. Svedberg and Bill (1972) divided the corneal endothelium into several areas on the basis of findings obtained by scanning electron microscopy.
Freeze-fracture studies of rabbit corneal endothelium clearly show that the regular hexagonal pattern is lost when the fracture is made slightly below but parallel with the apical cell surface. This fracture plane is localized below the level of the belt desmosome. When the fracture plane is localized at the level of the attachment of the endothelial cell to Descemet's membrane, the insertion is observed to have an irregular pattern, resembling a piece of a jigsaw puzzle. The basal surface of the endothelial cells shows a complex pattern of interdigitation (Hirsch et al. 1977), and the same applies to the lateral cell walls. A similar interdigitation of the lateral cell walls is found in man (Jakus 1961; Kuwabara 1978; Fine and Yanoff 1979; Binder et al. 1980).

2.3. PHYSIOLOGY

The stroma is made up of tidily arranged collagenous fibres. The periodicity of this endothelial collagen is 64-66 nm (Jakus 1961) and is typical of collagen. Several fibres together form a band, and these bands are in turn interlocked and together form what may be described as an internal skeleton of the cornea. The physicochemical properties of the corneal collagen are identical to those of cutaneous or tendinous collagen (Waltman 1981). The spaces between the corneal collagenous fibres are filled with a ground substance which consists of glucosamine glycans (mucopolysaccharides), proteins and keratocytes. The glucosamine glycans account for 4-4.5% of the dry weight of the cornea (Waltman and Kaufman 1970). These glycans can be subdivided into three groups: keratin sulphate (50%), chondroitins (25%) and chondroitin sulphate A (25%).
The glucosamine glycans are not found in the sclera. It is therefore logical to associate these substances with the clearness of the cornea (Waltman and Kaufman 1970). The chondroitin fraction is found exclusively in the cornea. The cornea, as an important part of the refractory system, should be clear and transparent. The clearness of the cornea is largely determined by the collagen structure on the one hand, and by an appropriate degree of hydration on the other.

The collagenous fibres are so arranged as to interfere as little as possible with the passage of light rays through the cornea. Light scatter is minimized by a proper spacing of the collagenous fibres. When these fibres are spaced less than one wavelength apart, the cornea remains clear because the scattered light rays extinguish each other by interference (Maurice 1957, 1960). When the fibres are spaced further apart, extinguishing interference no longer occurs. In fishes, the regular structure of the collagenous fibres of the cornea seems to play a less important role (Goldman and Benedek 1968).

The collagenous fibres themselves are embedded in a ground substance which contains glucosamine glycans. It is a typical characteristic of glucosamine glycans that they are able to behave like anions. This enables them to bind cations and, indirectly, water. The entire ground substance can therefore be regarded as a lyophilic colloidal system, which is subject to various physical and chemical influences (Waltman 1981).

The mechanism by which the collagenous fibres are tidily arranged and properly spaced (thus ensuring optimal optic function) is exceedingly complex. Under optimal conditions the stroma has a particular degree of dehydration. A regulatory system must ensure that the amount of fluid which is removed from the cornea equals the amount of fluid which enters it.

Beside the lyophilic colloidal system, electrolytic and osmotic balances between the cornea on the one hand and aqueous humour and tear fluid on the other play an important role in this regulatory mechanism (Mishima and Hedbys 1967). Both the epithelium and the endothelium play a role in maintaining an appropriate degree of corneal dehydration.

Intactness of these two layers is of importance for the optimal optical function of the cornea. This applies in particular to the endothelium. Lesions of the endothelial layer exert more influence on corneal hydration than lesions of the epithelium (Maurice and Giardini 1951). Evaporation of water on the epithelial side of the cornea plays an important role in the regulatory mechanism by which the proper degree of dehydration of the rabbit cornea is maintained (Von Bahr 1956; Mishima and Maurice 1961). This evaporation also plays a role in the human cornea. In patients with epithelial-endothelial dystrophy (cornea guttata, Fuchs' dystrophy), stromal oedema increases when the eyes have been closed for a considerable time, e.g. during sleep, and evaporation has been impaired by the closed eyelids (Cogan 1949). Apart from all the abovementioned factors, intraocular pressure also influences the degree of hydration of the corneal stroma.

The influence of the endothelium on the regulatory mechanism can be divided into an energy-independent (passive) and an energy-dependent (active) system. The passive system of regulation prevents an unlimited free influx of fluid to the stroma. This influx of fluid, small particles and nutrients to the stroma takes its course via the intercellular spaces (Kaye and Pappas 1962; Kaye et al. 1962; Rehm and Spagler 1976). This passive barrier function is correlated to

intactness of the tight junctions. Experiments in which calciumfree fluid was introduced into the anterior chamber, have demonstrated that the tight junctions are functionally damaged, with oedema of the corneal stroma as a result (Kaye et al. 1968, 1973, 1973a). This damage is reversible to a certain extent.
The passive barrier funtion can be measured in vivo with the aid of fluorescein as marker (Waltman and Kaufman 1970; Mishima 1975). In a group of patients with endothelial-epithelial dystrophy (cornea guttata, Fuchs' dystrophy), this passive barrier function was found to be reduced (Ota 1975; Burns et al. 1981); and the same was found in a group of patients given a corneal graft (Sato 1978).
The endothelium also plays an active role in removing fluid from the stroma (Maurice 1972). This process is based on pinocytosis (Kaye and Pappas 1962; Kaye et al. 1962). The pump mechanism utilizes ATP as source of energy. In addition to the Na-K pump mechanism, the bicarbonate ion plays an important role (Hodson 1977; Waltman 1981). This pump mechanism can be influenced by medication and by a decrease in temperature. In both cases the result is that not enough fluid is actively removed from the stroma with, as a result, stromal oedema.
The effect of the temperature is entirely reversible, provided sufficient glucose is present during the decrease in temperature. Once the proper corneal temperature is restored, dehydration is normalized (Harris 1960). The efflux of fluid from the cornea also removes metabolic waste products from the stroma. This (briefly described) complex regulatory mechanism to ensure the appropriate degree of corneal dehydration is required for an optimal optical function of the cornea. The regulatory role of the corneal endothelium is of central importance in this respect. Any lesion of the vulnerable endothelial layer sooner or later has its effect on the complex regulatory mechanism described. Lesions of any importance are bound to give rise to reversible or irreversible corneal oedema due to loss of this regulatory function of both the passive and the active part of the endothelial layer.

2.4 WOUND HEALING

Before discussing wound healing in general and corneal endothelial wound healing in particular, a brief survey of the cell cycle should be presented.

2.4.1 Cell cycle

The genetic codes of the cell are stored in the DNA. They are bundled to chromosomes and localized in the cell nucleus. In a population of dividing

cells, the chromosomes can be observed in a number of cells in a particular phase, i.e. the phase of nuclear division. Two phases of this type are separated by a period known as interphase.

When a cell divides, the daughter cells are identical to the mother cell. To ensure this, all genetic codes stored in DNA material have to be duplicated. At the same time the cytoplasm and the cytoplasmic organoids must be duplicated, and in this respect the messenger RNA and the RNA stored in ribosomes-play an important role. Cell division (cytokinesis) is preceded by nuclear division (karyokinesis or mitotic division).

In cell divisions in higher animal species, duplication of the DNA material takes place first and is followed by nuclear division. The normal diploid cell temporarily becomes tetraploid. After nuclear division the two nuclei are diploid again.

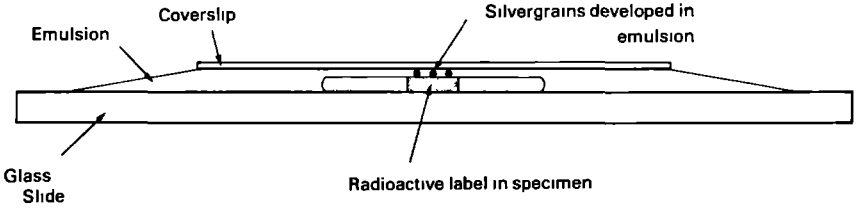
For detailed studies of the process of cell division, autoradiographic techniques are used (Hughes 1958; Leblond et al. 1959) which usually make use of radioactive thymidine. Both DNA and RNA possess four nucleosides. The two nucleic acids have three nucleosides in common: adenine, guanine and cystosine. The fourth nucleoside of DNA is thymidine, while that of RNA is uracil.

The period between two mitoses is known as interphase. In autoradiographic research investigating the interphase, tritium thymidine is offered to the cell culture or test animal. The cells to be studied take this labelled precursor up and incorporate it in the duplicated DNA. The test material is placed on a slide, fixed and then dipped in a liquid film emulsion. The film emulsion is thus exposed to the radioactivity incorporated in the DNA material. After development, the exposed parts of the film emulsion become visible as black dots (fig. 5).

When the material has been after-stained, the location of the radioactive material in the cell can be studied. In the case of DNA synthesis, black dots are localized above the nucleus. The resolving power at the range of radiation of tritium thymidine is about 1-3 μ (Leblond et al. 1959), and this is sufficient to study the incorporation of tritium thymidine at the cellular level.

Apart from the interphase, the four principal chromosomal configurations

FIG. 5 *Schematic representation of autoradiography. The film emulsion is exposed to the radioactive material in the tissue examined, beneath the film emulsion.*



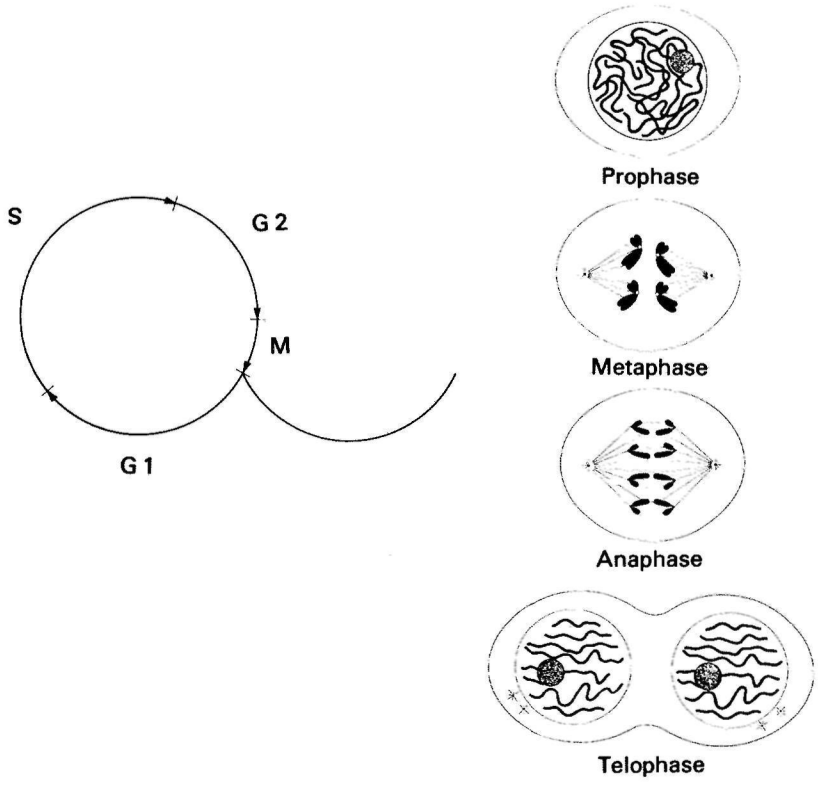


FIG. 6 *Schematic representation of the cell cycle. G-1 is the first gap phase, S is the DNA-synthesizing phase, G-2 is the second gap phase. M is the mitotic phase, which is subdivided into prophase, metaphase, anaphase and telophase, in chronological order. The chromosomal configurations during the various subphases of the M phase are represented schematically.*

which occur during the mitotic phase were already known. The above described autoradiographic technique revealed that the interphase can be divided into three phases (fig. 6).

The central phase of the interphase is the DNA-synthesizing phase (S phase); it lies between two phases in which no DNA synthesis takes place during autoradiographic studies: the so-called gap phases. The first gap phase (G-1) lies between the mitotic phase (M phase) and the S phase. This G-1 phase can be subdivided (into G-0 and G-1), but for the sake of simplicity this subdivision will be omitted. In the G-1 phase the cell is diploid. The second gap phase (G-2) lies between the S phase and the M phase. During this phase, no DNA is synthesized either. In this phase the cell is tetraploid.

In the higher animal species, most cells not involved in the mitotic cycle are in the G-1 phase, and diploid (Leuchtenberger et al. 1954).

The cells can be classified by various methods, one of which is based on their

cell cycle and ability to leave the G-1 phase and enter the S phase (Ham en' Cormack 1979). This classification distinguishes the following groups.

Group 1. Cells which are always in the G-1 phase. No stimulus of any kind can induce them to leave the G-1 phase and enter the S phase. An example of this cell type is found in the nerve cells.

Group 2. Cells which under normal conditions are in the G-1 phase and constitute part of a grown organ. Under these conditions these cells show but little spontaneous mitotic activity. When the organ is damaged, however, these cells leave the G-1 phase and enter the S phase, begin to synthesize DNA and complete the cell cycle. This process is arrested when the normal parenchymal mass of the organ is restored. An example of this cell type is the liver cell.

Group 3. Cells which are constantly in a cell cycle. One daughter cell differentiates and assumes a specific function, while the other daughter cell remains in the mitotic cycle as stem cell. Examples of this cell type are the germinitive epithelial cells and the stem cells of the erythropoietic and myelopoietic series.

It can be maintained in general that in higher animal species the S phase is followed by the M phase (Ham and Cormack 1979; Karp 1979). However, exceptions to this rule have been described.
Gelfant (1963) demonstrated that some mouse epidermis cells are in the G-2 phase. He established this by determining the DNA content per cell. He assumed that these cells might function in a primary restorative mechanism. The cells can divide immediately after damage, without first synthesizing DNA, and thus contribute to accelerated wound healing.
Other types of division, which do involve karyokinesis but not true cell division (cytokinesis), are found exclusively in plants and primitive animal species (Prescott 1976). This type of cell division proves to play no role in animal cells, with the possible exception of tumour cells. Other types of DNA synthesis were observed and studied especially in plants (Prescott 1976). In higher animal species, no nuclear division takes place until DNA synthesis is first completed. The only exception is the formation of haploid sex cells (meiosis), which involves true nuclear division and cell division without previous duplication of the DNA material. After fusion of the gametes, a diploid cell forms.
Under certain conditions DNA synthesis can take place outside the S phase, e.g. to restore damaged DNA after ultraviolet irradiation or exposure to cold (Rasmussen and Painter 1966; Pelc and Viola-Magni 1969). DNA synthesis was demonstrated in human corneal endothelium after ultraviolet irradiation (Grabner and Brenner 1981).

When a test animal is injected with tritium thymidine, the thymidine not incorporated in DNA is quickly broken down after the injection, and its excretion is virtually complete within an hour (Leblond et al. 1959; Potter 1959; Rubini et al. 1960; Claever 1967), whereas re-incorporation of thymidine is only limited under normal conditions (Bryant 1962; Diderholm et al. 1962). Injection of tritium thymidine into the anterior chamber of the rabbit eye is followed by its exponential removal from the aqueous humour. The concentration demonstrable after an hour is only 10% (Maenza and Harding 1962).

2.4.2 General introduction to wound healing

Wound healing has been studied in detail especially in the epithelium of the skin. The healing process which occurs after a skin lesion takes its course in accordance with a particular reaction pattern. In principle, this complete reaction pattern is observed in al types of epithelium.
In the skin - a stratified epithelium - the wound begins to fill with blood immediately after the trauma (Pollack 1979; Haferkamp 1980). A fibrin network is formed which seals the wound. The morphology and function of the adjacent vital epithelial cells change within 12 hours. The cells become flatter, cell junctions are in part disrupted, and the cells arrange themselves in a less tight pattern, so to speak (Ortonne et al. 1981).
The cells start to show ruffled borders due to pseudopodium-like cytoplasmic processes. The cytoplasm itself is also subject to changes, and filaments with a diameter of 4-8 nm begin to be formed. These filaments, which selectively bind anti-actin antibodies, are observed only during the healing process.
These findings suggest that this apparatus of contractile microfilaments enables the cells to migrate. This migration takes place in a manner reminiscent of the amoeba. Once these changes have occurred in the cytoplasm, the normal specialized function of the cell ceases. The normal specialized function of the epithelial cell is keratin formation.
The cell develops new activities which are required for the process of migration, and prepares itself for DNA synthesis. The mechanism of this activation is still obscure. The loss of cell junctions (and consequently possibly the disappearance of 'contact inhibition') seems to be one of the principal factors involved. Once the migrating and dividing epithelial cells have covered the wound, they form new cell junctions and resume their original morphology and physiology (Ortonne et al. 1981). Mitotic activity is maximal 48 hours after the trauma, and rapidly diminishes as soon as the wound is epithelialized. Fibroblasts need more time to migrate into the wound (about 72 hours). Collagen synthesis begins on the 5th day. This first period of 5 days is known as lag phase; this phase is characterized by epithelialization but shows no collagen synthesis as yet.

The phase in which collagen synthesis takes place is known as fibroplastic phase. The maximum of collagen synthesis is seen on the 7th day. The amount of collagen formed is dependent in part on the force applied to the wound (Pollack 1979).
The primarily synthesized collagenous fibres are still arranged at random. Their re-arrangement is determined by forces which act on the wound. Once water and glucosamine glycans have left the wound, the collagenous fibrils are compressed, and this leads to cross-linking and consequently more intensive interfibrillar contact.
Lower animal species reduce the surface area of the wound in order to effect more rapid epithelialization. They achieve this by contractions of subcutaneous muscle fibres which form the panniculus carnosus. Man possesses only a vestige of the panniculus carnosus; moreover, the human skin is not sufficiently mobile to be effective in this aspect of wound healing.
Modified fibroblasts are found in the granulation tissue of open wounds (Rudolph 1979). These cells contain contractile proteins. These myofibroblasts reduce the wound surface area by contraction, so that re-epithelialization can take place as quickly as possible. Myofibroblasts are found in granulating wounds and, to a lesser extent, in well-adapted and closed wounds (Ryan et al. 1974). The myofibroblast shares characteristics with both the fibroblast and the smooth muscle cell. It is therefore able to synthesize collagen as well as to contract. Collagen as such cannot contract. It is passively moulded to a certain shape by contraction of the myofibroblast and consolidates in that shape while the cross-linking takes place. In phylogenetic terms, the myofibroblast would seem to compensate the absence of a panniculus carnosus.
However, many other factors also influence this (briefly described) wound healing process (Pollack 1979a, 1979b). The general principles of wound healing apply to all kinds of epithelial lining. In this respect the cornea differs from the skin only by its avascularity. However, the aqueous humour assumes the function which the blood supply has in the healing of skin lesions.

2.4.3 Corneal wound healing

The monolayers which line the blood vessels, lymphatics and serous cavities are histologically regarded as simple squamous linings (Bloom and Fawcett 1975). Meier (1977) regards the endothelium as a posterior corneal epithelium.

2.4.3.1 Endothelium

In the young adult rabbit - the test animal most widely used in research concerning the cornea - surrounding vital endothelial cells begin to migrate

into the wound after an endothelial injury. This process commences within 5-7 hours (Faure et al. 1971; Sperling 1978; Binder et al. 1980). Specular microscopy (Maurice 1968; Bourne et al. 1976) makes it possible to observe that this process commences more rapidly in vivo (Sherrard 1976; Olson et al. 1978).

It seems as if the vital endothelial cells start to form pseudopodia which enable them to migrate into the wound area by means of an amoeboid movement (Binder and Binder 1957; Chi et al. 1960; Polack 1975; Khodadoust and Green 1976). During this process the basal cell surface area increases, and the cells become flatter. The formed cytoplasmatic processes give the endothelial cells a fibroblast-like appearance (Binder and Binder 1957; Chi et al. 1960; Stocker 1971; Michels et al. 1972; Matsuda and Smelser 1973, 1973a; Polack 1975; Doughman et al. 1976; Kenyon et al. 1976; Khodadoust and Green 1976; Van Horn et al. 1977; Fine and Yanoff 1979; Silbert and Baum 1979; Waller 1980; Renard et al. 1981). This change in the morphology of endothelial cells is observed also in graft reactions of corneal grafts (Polack 1977).

In rabbits, the endothelial wound is re-endothelialized within 2-9 days, depending on its size. Long cell processes of migrating endothelial cells cross, but the cells nevertheless remain in contact. During the process of cell migration the cell processes gradually diminish in size, and the cell bodies come closer together again. When the wound is covered with endothelium, the endothelial cells lose their fibroblast-like appearance (Matsuda and Smelser 1973; Waring et al. 1974; Polack 1975; Van Horn and Hyndiuk 1975; Doughman et al. 1976; Khodadoust and Green 1976; Van Horn et al. 1977; Binder et al. 1980; Fujikawa et al. 1980; Renard et al. 1981).

When the cell bodies are sufficiently close together again, new cell junctions can be formed (Hirsch et al. 1975). Desmosomes and tight junctions are formed within 5 days after infliction of the wound, and gap junctions form slightly later (Van Horn and Hyndiuk 1975; Hirsch et al. 1976; Renard et al. 1976).

Endothelial function is usually restored 4-5 days after histological restoration (Baum and Silbert 1978).

A similar process of formation of new cell junctions is observed in the healing process of liver and skin wounds (Loewenstein and Penn 1967; Loewenstein 1978).

The endothelial cells in the wound and in the area around it, show an irregular appearance (pleiomorphism), in contrast to the endothelium further away from the wound and not immediately involved in wound healing (Kara Jose et al. 1972). These more distant cells present their normal hexagonal pattern (Kara Jose et al. 1972; Yano and Tanishima 1980); and the wound also resumes a virtually normal appearance, but after a longer time (Tost et al. 1968; Hirsch et al. 1976; Khodadoust and Green 1976; Sherrard 1976; Yano en Tanishima 1980). This process of reallocation has been observed in man in

vivo with the aid of specular microscopy (Rao et al. 1978, 1980; Olsen 1981), and in test animals by means of scanning electron microscopy (Van Horn and Hyndiuk 1975; Van Horn et al. 1977), with silver staining (Hirsch et al. 1975, Yano and Tanishima 1980) and with nuclearstraining (Capella 1972).
It has been established in rabbits that, apart from the mechanism of migration described, the corneal endothelium is capable of proliferation as part of wound healing (Binder and Binder 1957; Morton et al. 1958; Chi et al. 1960; Mills and Donn 1960; Bito and Harding 1961; Von Sallmann et al. 1961; Von Sallmann et al. 1963; Chi and Kelman 1966; Sommerville and Shea 1966; Tost et al. 1968; Carreras and y Jordano 1974-1975; Hirsch et al. 1975; Renard et al. 1976; Van Horn et al. 1977; Fujikawa et al. 1980, Yano and Tanishima 1980). A correlation has been established between age and proliferative activity (Von Sallmann et al 1961; Oh 1963; Staatz and Van Horn 1980). Amitotic cell division and the development of giant cells have been observed in the process of endothelial wound healing (Faure et al. 1971).
Primates, including man, are assumed to differ from the rabbit in that corneal endothelial wound healing in these species involves only the mechanism of cell migration (Van Horn and Hyndiuk 1975: non-human primate, scanning electron microscopy; Doughman et al. 1976: human corneas in vitro, scanning electron microscopy; Capella 1972: primate and rabbit, nuclear staining of a flat endothelial specimen; Renard et al. 1981: human corneas scanning electron microscopy; Kaufman et al 1966: human corneas, nuclear staining of a flat endothelial specimen; Kaufman and Katz 1977: human corneas, specular microscopy; Stocker 1971: non-human primate, cross-sections).
Precisely because there is no proliferation of corneal endothelium in monkeys and cats, these animals should be more suitable as test animals to imitate the human situation (Van Horn et al. 1977; Gospodarowicz et al. 1979).
Weimar et al. (1980) found that acceleration of corneal endothelial wound healing could be induced in cultured rabbit corneas with mesodermal growth factor. It was also found possible to stimulate the healing of corneal endothelial wounds in cultured bovine corneas with fibroblast growth factor (Gospodarowicz and Greenburg 1979). Autoradiographic techniques were used to demonstrate that the corneal endothelium of rats and frogs is able to synthesize DNA (Gordon and Rothstein 1978).
In rats, healing of an endothelial wound in the aorta involves cell migration in the initial phase, which is subsequently supported by proliferation (Schwartz et al. 1978).
In-vitro experiments with cultured human corneas yielded indications which were at odds with the currently accepted view that the corneal endothelium of primates, including man, is incapable of proliferation. Experiments with cultured human corneas revealed accelerated healing of endothelial wounds after stimulation with mesodermal growth factors. Both mitotic and amitotic

cell division patterns were observed in the wounds (Squires and Weimar 1980). Fabricant et al. (1981) demonstrated that human corneal endothelium contains receptors for epidermal growth factors. Zagorski (1980) observed mitotic figures during human corneal endothelial wound healing not stimulated with growth factors. The culture medium, however, did contain calf serum.
DNA synthesis has been demonstrated as a feature of corneal endothelial wound healing in cultured human corneas (Simonsen et al. 1981) and in vivo in monkeys (Gloor et al. 1980). Wolter (1967) described proliferation of human corneal endothelium in histopathological sections after purulent keratitis and ulceration. But he did not describe mitotic figures.
An age correlation was established in the successful tissue cultering of human corneal endothelial cells (Baum et al. 1979).
A study of wound healing in confluent monolayers of cultured human endothelial cells from umbilical blood vessels reveals that a process of migration starts within 12 hours after infliction of the wound. Wound healing is supported by proliferation of endothelial cells after 36 hours. The start of the process of proliferation can be inhibited by exposing the endothelial culture to irradiation with 1500 Rad. one hour before the wound is inflicted. This irradiation exerts no influence on the process of migration (Sholley et al. 1977).

2.4.3.2 Stroma

Immediately after infliction of a wound the defect is filled with a fibrin plug (Binder et al. 1980; Goder 1980). The keratocytes in the immediate vicinity of the wound degenerate completely. The wound area becomes oedematous. The keratocytes at the margin of the wound area are transformed and assume a fibroblast-like appearance. This transformation takes place 12-24 hours after infliction of the wound (Binder et al. 1980). The fibroblasts migrate into the wound area and reach the stromal defect proper after about 2-3 days. This is when the production of glucosamine glycans and collagen begins. The keratocytes further away from the wound present a normal appearance.
The peak of protein synthesis and of proliferative activity occurs between the 5th and the 14th day. After this period, proliferation and protein synthesis gradually diminish and the stromal wound slowly attains its ultimate strength. Proliferation can be accelerated by application of growth-stimulating substances (Smith et al. 1981).

2.4.3.3 Combined endothelial and stromal wound

In a combined endothelial and stromal wound, individual wound healing as

described in paragraphs 2.4.3.1 and 2.4.3.2 begins simultaneously. Other, additional factors now also play a role as determinants of the ultimate result. Wound adaptation in the deeper layers plays an important role. In particular the deeper layers of the stromal wound form a major barrier in endothelial wound healing because Descemet's membrane is absent at this level (Hinzpeter and Naumann 1977; Gospodarowicz and Ill 1980). The importance of the proper substratum for migrating cells was demonstrated by Yaoi (1972) and Yaoi et al. (1972).

Human corneal endothelium is capable of migrating across a stromal wound. This was observed in wound healing after aqueous humour invasion in a patient with keratoconus (Stone et al. 1976).

Good adaptation of the deeper wound layers should be followed by rapid re-endothelialization (Heller et al. 1971; Sanchez et al. 1974; Eve and Troutman 1976). An undisturbed course of this process also contributes to reduction of late complications after corneal grafts (Witmer 1980). It enables the intact endothelial layer to resume its function: regulation of the degree of corneal dehydration. The oedema in the wound area consequently decreases, and synthesis of Descemet material is resumed. A new Descemet membrane is then deposited over the deep stromal wound. (Smelser and Ozanics 1972; Leuenberger et al. 1973; Waring et al. 1974; Yue et al. 1978).

The proliferative activity of the stromal component diminishes as soon as re-endothelialization is completed (Rycroft 1965; Brown and Kitano 1966; Sherrard and Rycroft 1967, 1967a; Kurz and D'Amico 1968; Sherrard 1969; Smith 1970; Werb 1972; Lemp 1976; Hinzpeter and Naumann 1977; Sanchez and Polack 1978).

An inhibitory effect of re-endothelialization of an endothelial wound has also been observed in the intima of blood vessels (Haudenschild and Schwartz 1979).

Poor adaptation of deeper wound layers impedes rapid re-endothelialization the more so when many endothelial cells have been lost in the trauma. The number of endothelial cells lost around the stromal wound in an important determinant of the ultimate result (Moore and Aronson 1973). In such cases, no inhibitory effect on the granulation process of the stromal wound healing components occurs by the re-endothelialization, and consequently this granulation tissue contributes to the formation of a retrocorneal membrane (Werb 1972; Lemp 1976; Hinzpeter and Naumann 1977). Histopathological examination of unsuccessful corneal grafts nearly always reveals a disturbance in the deeper layers of the cornea, and often also retrocorneal membranes (Chi et al. 1962; Hales and Spencer 1963; Rycroft 1965; Brown and Kitano 1966; Kurz and D'Amico 1968; Winter 1969; Smith 1970; Kanai et al. 1972; Hinzpeter and Naumann 1977; Knöbel and V. Damarus 1979; Lahav and Cadet 1979).

A combined wound in fact involves a degree of competition of two wound healing processes: that of the lining tissue and that of the underlying tissue.

The lining tissue must fight against time, so to speak. The crucial question is: can it cover the wound surface before the granulation process of the underlying tissue disturbs the ideal healing process? A similar competition between two types of tissue is observed in the healing of a peripheral nerve lesion (Ham and Cormack 1979).

For a better understanding of postoperative complications after a corneal graft, a better understanding of the corneal wound healing process is required. More specifically the healing process in the deeper wound layers was studied in this thesis. In adult monkeys, the peroperative endothelial cell loss during a corneal graft procedure was studied. In the subsequent wound healing process, the deeper wound layers were specifically studied. Next, the components of the healing process of a purely endothelial wound were studied. This was done in adult monkey corneas in vivo, in human corneas in vitro, and in human corneas in vivo.

MATERIAL AND METHODS

3.1 MATERIAL

The study was divided into three parts:
In-vivo corneal endothelial wound healing in non-human primates (3.1.1).
In-vitro human corneal endothelial wound healing (3.1.2), studied in 152 corneas.
In-vivo human corneal endothelial wound healing (3.1.3), studied in two patients.
Parts 3.1.2 and 3.1.3 were carried out at the Bascom Palmer Eye Institute, Department of Ophthalmology, University of Miami School of Medicine, Miami, Florida, USA (Head: E.W.D. Norton, M.D., Professor and Chairman).
For part 3.1.3 we obtained permission from the Medical Sciences Subcommittee (R-64) for the protection of human subjects, office of research administration, School of Medicine, University of Miami, Florida (protocol number 81/10).

3.1.1 In-vivo corneal endothelial wound healing in non-human primates

Nine primates *(Macaca mulatta)* were used in this part of the study (see table 3.1.1). They had been captured as young wild adults and had since been kept for at least 9 years at the animal laboratory of the University of Nijmegen. At the time of the experiments the animals must have been 11-14 years old. The maximum age reached by this species averages 25-30 years, and the monkeys used can therefore be described as primates of adult to middle age.
All animals used in the study were sacrificed by means of an overdose of barbital upon completion of the experiments.

TABLE 3.1.1 *In-vivo corneal endothelial wound healing in non-human primates*

3.1.1.1 Endothelial loss and wound healing after corneal allograft.
3.1.1.2 Endothelial wound healing.
3.1.1.3 Endothelial wound healing after corneal autograft.

Autoradiography of the flat corneal endothelial specimens was performed in groups 3.1.1.2 and 3.1.1.3. For this purpose, 50 μl fluid containing 10 μCi tritium thymidine (spec.ac. 26 Ci/mmol, Amersham) was injected into the

anterior chamber of the eye with a 30 gauge needle. This was done under general anaesthesia, 1 hour before completion of the experiment. To prevent leakage of radioactive material, the fluid was slowly injected in the course of two minutes. The experiment was completed when the tritium thymidine had been in contact with the wound for 1 hour.

3.1.1.1 Endothelial loss and wound healing after corneal allograft

In this group of four monkeys (74, 75, 87 and 98), a 5 mm corneal graft was performed, unilateral in two animals and bilateral in the other two (see table 3.1.1.1). The operation was performed under general anaesthesia with pentobarbital, nitrous oxide and oxygen, and intubation anaesthesia.

Donors:
The donor material was obtained from the same species. The donor animals were sacrificed to obtain the tissue required for virus culture (National Institute of Public Health, Utrecht).
The eyes were enucleated and immediately sent to the animal laboratory of the University of Nijmegen. During transport the temperature of the container with the enucleated eyes was kept constant at 4°C. In this way the donor tissue could be transplanted within 6 hours in all cases. After arrival the cornea with a scleral rim of 2 mm was removed from the eyeball, and a 5 mm graft was punched out from the endothelial side.

Grafting technique:
After bridling of the superior and inferior rectus muscles, a central corneal disc with a diameter of 5 mm was removed by trephination and cutting. The donor disc was sutured in situ with 4 stitches (Alcon 10-0). The graft was definitively fixed in position with a continuous suture (Alcon 10-0).
This continuous suture was placed immediately above Descemet's membrane, without pearcing it. To prevent irritation from the knot, this was buried beneath the conjunctiva after adequate tautening and knotting of the continuous monofil. During the making of the continuous 10-0 suture the 4 situation stitches were removed. At the end of the operation the anterior chamber was brought to the proper depth with a balanced salt solution, making sure that no anterior synechiae were present. A Zeiss operation microscope was used. A single application of chloramphenicol eye ointment completed the operation.
At the end of the period of observation (see table 3.1.1.1) the animal was sacrificed and both eyes were removed immediately. The endothelial defects were studied after removing the entire cornea with a scleral rim of 2 mm from the eyeball and staining the endothelium with a 0.5% trypan blue solution

(vital stain) during 90 seconds. The endothelial lesions were photographed, whereupon the cornea was fixed in 2% glutaric aldehyde at 4°C for further examination with the scanning electron microscope.
To minimize dehydration during photography, the cornea was regularly moistened by immersion in a balanced salt solution. Despite these precautions an occasional dehydration artefact could not be prevented.
For light-microscopic examination a corneal segment was so cut off that a part of the graft was included, and processed.
The corneas of the two untreated eyes were removed and stained with silver, whereupon a flat endothelial specimen was prepared. These corneas served as controls for parts 3.1.1.2 and 3.1.1.3 of the study. The inner surface of the cornea was studied 0, 0, 0, 4, 8 and 28 days after the operation, respectively (see table 3.1.1.1).

TABLE 3.1.1.1 *Non-human primates given a 5 mm corneal graft. OD = right eye. OS = left eye.*

Number	Corneal graft	Period of observation in days	Processing technique		
			Trypan blue	SEM*	Cross-sections
74 OD	x	8	x	x	x
74 OS	control				
75 OD	x	28	x	x	x
75 OS	x	0	x	x	x
87 OD	x	4	x	x	x
87 OS	x	0	x	x	x
98 OD	x	0	x	x	x
98 OS	control				

* Scanning Electron Microscopy

3.1.1.2 Endothelial wound healing

For further study of the wound healing pattern observed in 3.1.1.1, two further experiments were performed to establish whether the endothelial healing involved DNA synthesis as an expression of proliferation. For this purpose the silver staining was combined with autoradiographic examination (see table 3.1.1.2).

Mechanical lesion:
In this group of two monkeys (81 and 82), the question whether the endothelial wound healing involved DNA synthesis was studied. A limbal incision was made in the cornea under general anaesthesia (for technique, see 3.1.1.1). A curved lacrimal probe with a diameter of 1 mm was inserted into the anterior chamber. The tip of the probe was used to produce an endothelial lesion in the central part of the cornea, whereupon the incision was closed with two stitches (Alcon 10-0). A single application of chloramphenicol followed.

TABLE 3.1.1.2 *Non-human primates in which the healing of a mechanically induced corneal endothelial wound was studied. OD = right eye Os = left eye*

Number	Mechanical wound	Period of observation in days	Processing technique	
			Silver staining	Autoradiography
81 OD	x	3	x	x
81 OS	x	1	x	x
82 OD	x	2	x	x
82 OS	x	0	x	x

The animals were sacrificed and the eyes removed, whereupon the cornea with a scleral rim of 2 mm was dissected out and stained with silver. Autoradiography of the flat specimen followed. Specimen 81 OS was after-stained with methyl green/pyronine (McMannus and Mowry 1960). Wound healing was studied after 0, 1, 2 and 3 days (see table 3.1.1.2).

3.1.1.3 Endothelial wound healing after corneal autograft

In this group of 3 monkeys (72, 78 and 89), a 5 mm corneal graft was performed under general anaesthesia as described in 3.1.1.1 (see table 3.1.1.3). A central corneal disc was trephined and dissected free, and then stitched in position again. The operative technique was the same as that described in 3.1.1.1.

TABLE 3.1.1.3 *Non-human primates given a 5 mm corneal autograft OD = right eye OS = left eye*

Number	Corneal graft	Period of observation in days	Processing technique	
			Silver staining	Autoradiography
72 OD	x	2	x	x
72 OS	x	6	x	x
78 OD	x	3	x	x
78 OS	x	0	x	x
89 OD	x	13	x	x
89 OS	x	1	x	x

The animals were sacrificed at the end of the period of observation and both eyes were removed, whereupon the cornea with a scleral rim of 2 mm was dissected out. The cornea was stained with silver, and this was followed by autoradiography of the flat corneal specimen. Wound healing was studied after 0, 1, 2, 3, 6 and 13 days.

3.1.2 In-vitro human corneal endothelial wound healing

All corneas were obtained by courtesy of the Florida Lions Eye Bank, Miami (Med. director: V T. Curtin, M.D.), within 24 hours of death. The donor age ranged from 1 to 76 years (for specific data, see section 2 of chapter 4). The corneas were used in nine different experiments (see table 3.1.2)

TABLE 3 1 2 *Human corneas in which the healing of an endothelial wound was studied*

Experiment	Number of corneas	Purpose	Silver staining	Auto-radio-graphy	Nuclear staining
3 1 2 1	14	Standardization of endothelial wound	x		
3 1 2 2	11	Controls	x	x	x
3 1 2 3	20	DNA synthesis?	x	x	x
3 1 2 4	44	DNA synthesis and/or mitosis	x	x	x
3 1 2 5	10	Autoradiographically positive mitotic figure		x	x
3 1 2 6	20	Qualitative difference in wound healing between a mechanical and a cryo-induced corneal endothelial lesion	x	x	x
3 1 2 7	16	Influence of exogenous factors	x	x	x
3 1 2 8	10	Scanning electron microscopy of wound healing			
3 1 2 9	7	Cross-sections			

Technique of resecting donor corneas:
First, the palpebrae of the donor were cleansed with an alcohol-soaked gauze under aseptic conditions; the superior and the inferior fornix were then washed with a balanced salt solution, whereupon a few eyedrops (Neosporine®) were applied.
The conjunctiva was then dissected free along the limbus. A razorblade was used to incise the sclera as far as the choroid, 2-3 mm posterior to the limbus, whereupon scissors were used to cut it loose all around. The anterior chamber was often still in position at this time. The scleral rim was grasped in forceps and cautiously pulled loose from the choroid and the scleral spur. The cornea was immediately transferred to a tissue culture jar which contained 10 ml modified Delbucco tissue medium.

Technique of producing a mechanical endothelial lesion:
To inflict the wound, the cornea was removed from the tissue culture jar and, with the endothelial side up, placed on a concave block of sterile teflon. A depression in the teflon block had a radius which corresponded with the average radius of the anterior corneal surface (r = 7.70). The stylet used to produce the lesion had a diameter of 3 mm and a rounded, polished tip. A special holder ensured that the stylet always hit the cornea perpendicularly and centrally (fig. 7).
An endothelial lesion of standard size was produced by allowing the stylet to rest its weight on the cornea and carefully giving it a few turns. This was considered necessary if the autoradiographic results and the wound healing at

FIG. 7 *Special holder with turnable stylet used to produce a standard lesion of the corneal endothelium. Insert: Detail of the polished tip and the teflon block.*

different times were to be compared. This is why a mechanical wound technique was preferred to a transcorneal cryo-lesion, which does not permit a comparison in time because there is no standard wound size.
The endothelial lesion was produced under aseptic precautions in an laminar flow cabinet. The cornea was then returned to the culture jar and placed in the incubator. The culture was made at a temperature of 37°C, a relative humidity of 98% and a carbon dioxide concentration of 5%.
For autoradiographic studies tritium thymidine (specific activity 6.7 Ci/mmol, New England Nuclear Corporation) was added to the culture medium to an ultimate concentration of 5 μCi/ml (unless differently specified). Depending on the type of experiment, the tritium thymidine was added at the start of the experiment (continuous fashion) or 1 hour before its completion (pulsed fashion).
In a number of cases (13 corneas) the cornea was placed in a fresh culture medium containing unlabelled thymidine at the end of the experiment. This was done in order to establish whether non-specific reversible binding of the tritium thymidine to the endothelial cells had occurred. For this purpose the corneas were placed in the fresh medium for 15 minutes, whereupon further processing took place. All corneas were fixed in 5% glutaric aldehyde at 4°C.
This study of in-vitro human corneal endothelial healing was divided into nine experiments (see table 3.1.2).

3.1.2.1 Standardization of endothelial wounds

The above described equipment was used to produce a mechanical lesion in 14 corneas. The corneal specimen was immediately stained with silver. The size of the lesion was then measured in order to establish whether the lesion was reproducible and whether Descemet's membrane had remained intact.

3.1.2.2 Controls

Eleven corneas were cultured without a central endothelial lesion. The culture medium contained tritium thymidine (5 μCi/ml) from the start of the experiment. These corneas served as controls for subsequent experiments. They were examined after 2, 3 and 4 days.

3.1.2.3 DNA synthesis?

A central endothelial lesion was mechanically produced in 20 corneas, tritium thymidine (5 μCi/ml) having been added to the medium at the start of the

experiment. The purpose of this experiment was to establish whether DNA synthesis of endothelial cells occurred as a feature of endothelial wound healing. The period of observation was 2, 3, and 4 days.

3.1.2.4 DNA synthesis and/or mitosis

In this experiment, a standard lesion was mechanically produced in 44 corneas. One hour before completion of the experiment, tritium thymidine (5 μCi/ml) was added to the culture medium. During this hour, all cells which would synthesize DNA were labelled. In this way an impression was gained of the time interval between wound healing and DNA synthesis. The non-labelled cells could be examined for mitotic figures with the aid of nuclear staining. The corneas were examined after 1, 2, 3 and 4 days.

3.1.2.5 Autoradiographically positive mitotic figures

A standard lesion was mechanically produced in 10 corneas, which were then cultured for 3 days. Tritium thymidine was added to the culture medium (1 μCi/ml) at the start of the experiment. These corneas were examined only by autoradiography and nuclear staining. The experiment was performed in order to demonstrate a mitotic figure with a positive autoradiograph. The lower tritium thymidine concentration ensured a less intensive density of the autoradiograph, which facilitated evaluation of the nuclear stain.

3.1.2.6 Qualitative difference in wound healing between a mechanical and a cryo-induced corneal endothelial lesion

A standard endothelial lesion was mechanically produced in one cornea of each of 10 pairs of corneas, while in the other cornea of each pair a transcorneal cryo-lesion was induced. For this purpose the cornea was frozen (-60°C) for 20 seconds, rewarmed and again frozen (-60°C) for 20 seconds. The diameter of the cryoprobe was 2 mm. The purpose of this experiment was to establish whether a qualitative difference in wound healing existed between lesions produced by these two techniques. The experiment was also performed as a preparation for the study of in-vivo human corneal endothelial healing (3.1.3).

3.1.2.7 Influence of exogenous factors

A standard lesion was mechanically produced in 8 pairs of corneas. One

cornea of each pair was cultured in the medium described in subsection 3.2.1, while the other cornea was cultured in a medium to which no foetal calf serum had been added. This experiment was performed in an attempt to establish whether the results observed were or were not attributable exclusively to exogenous factors contained in the foetal calf serum.

3.1.2.8 Scanning electron microscopy of wound healing

The scanning electron microscope was used to study the healing of standardized endothelial wounds in 10 corneas. The purpose of this experiment was to establish whether this method of investigation would supply additional information if compared with the combined histological processing used in this study. Moreover, it ensured a different visualization of wound healing. The wounds were examined after 0, 1, 2, 3 and 4 days.

3.1.2.9 Cross-sections

In order to study the normal endothelial structure and the nature of the silver staining, a number of normal corneas were examined histologically and/or electron-microscopically. The corneas were fixed in their normal shape; no flat specimens were prepared. After fixation the cornea was cut into two halves. One half was embedded in paraffin and further processed for light microscopy, while the other half was embedded in Epon and processed for transmission electron microscopy.
One cornea was processed in the manner described in order to gain an impression of the morphology of its normal anatomy. Three corneas, stained with silver without an endothelial lesion, were examined histologically and electron-microscopically. Some sections were after-stained with haematoxylin and eosin.
Two corneas with an endothelial lesion of standard size were stained with silver after a wound healing period of 2 and 3 days, respectively, and examined histologically and electron-microscopically. Some sections were after-stained with haematoxylin and eosin.
One cornea was cultured for 3 days in a medium containing tritium thymidine (5 μCi/ml), and silver-stained at the end of the experiment. Wound healing was examined both histologically and electron-microscopically. The histological sections were autoradiographically examined as well. The sections were after-stained with haematoxylin.

3.1.3 In-vivo human corneal endothelial wound healing

Two patients gave their informed consent and volunteered for this part of the study, which concerned in-vivo human corneal endothelial healing (see table 3.1.3)

TABLE 3.1.3 *In-vivo human corneal endothelial wound healing.*

Number of corneas	Purpose	Silver staining	Autoradiography	Nuclear staining
2	in-vivo wound healing	x	x	x

Both patients (aged 65 and 67, respectively) were to undergo enucleation for other ophthalmological reasons. The central part of the cornea was frozen twice for 20 seconds with a cryoprobe with a diameter of 2 mm, 3 days and 5 days before the operation, respectively.
The temperature during application of the cryoprobe was -60°C. After rewarming of the cornea, the cryoprobe was again applied to the same area (comparable with experiment 3.1.2.6). The freezing was done under local anaesthesia after instillation of an analgesic (Alcaine®). An eye dressing was applied to the eye after the induction of the transcorneal endothelial lesion. No medication was given.
Immediately after enucleation of the eye, the cornea with a scleral rim of about 2 mm was removed from the eyeball and placed in the culture medium. Tritium thymidine had been added to the medium to a final concentration of 5 μCi/ml. The cornea was placed in the incubator for 1 hour, under the same conditions as in all in-vitro experiments. At the end of this hour the cornea was stained with silver, whereupon autoradiography and nuclear staining were carried out.

3.2 METHODS

The following techniques were used.
Organ culture (3.2.1).
Trypan blue (vital) staining (3.2.2).
Silver staining (3.2.3).
Preparation of a flat corneal endothelial specimen (3.2.4).
Autoradiography (3.2.5).
Nuclear staining (3.2.6).
Scanning electron microscopy (3.2.7).
Transmission electron microscopy (3.2.8).
Special techniques are described in the relevant sections and subsections.

3.2.1 Organ culture

The culture medium used in all experiments was Delbucco modified Eagles with addition, per litre of medium, of: 5% dextran, median molecular weight 40,000 (Sigma), 10% foetal calf serum (ultimate concentration) (Gibco Company), 6 g Hepes buffer (25 mmol), 1.25 mg amphotericin B, 100,000 U penicillin, and 100 mg streptomycin. Phenol red was used as pH indicator.

3.2.2 Trypan blue (vital) staining (Stocker 1971)

Trypan blue was dissolved in distilled water to an ultimate concentration of 0.5%. The endothelium was stained for 90 seconds, whereupon the excess trypan blue was washed off in physiological saline. The cornea was then immediately examined and photographed.
Only cells whose cell membrane is damaged stain immediately with trypan blue.

3.2.3 Silver staining (Smolin 1968)

After completion of the experiment the cornea is placed for at least 10 seconds in 10 ml of a 10.3% sucrose solution at 20°C. Next, the cornea is cautiously placed for 10 seconds in a 0.25% silver nitrate solution in 10.3% sucrose solution at 20°C, whereupon it is washed twice in a 10.3% sucrose solution at 20°C.
The cornea is then placed in a 10.3% sucrose solution with the endothelial side up and 1 cm below the fluid surface. The endothelium is then exposed to UV light from a source 5 cm above the fluid surface (Burton Lamp, Cavitron Urton 9110 bulb). The cornea is exposed for two minutes, whereupon light-brown discoloration of the endothelium is seen. The degree of silver staining can be assessed in a microscope. It can be itensified if necessary by prologing the time of exposure.
Exposure for two minutes was found to be generally sufficient, particularly if the correct temperature is carefully maintained. The specimen thus stained is studied in transmitted light in a regular microscope.

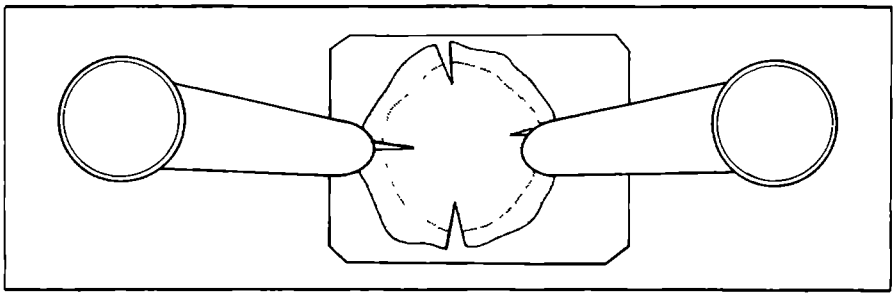
3.2.4 Preparation of a flat corneal endothelial specimen

After silver staining (if any) four radial incisions are made in the cornea with the aid of a razorblade (Nicholas 1965). They cause the cornea to lose its concave shape and more or less flatten it. When the cornea is fixed in this

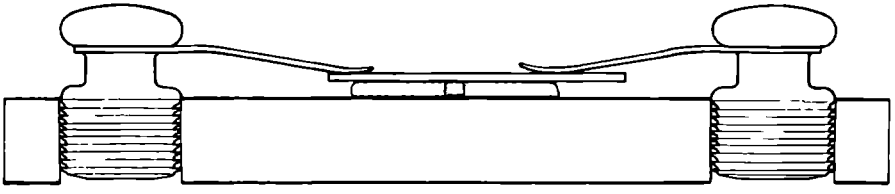
condition, no entirely flat endothelial specimen is obtained.
Other flat specimens prepared by removing the stroma (Soong et al. 1978) or the stroma together with Descemet's membrane (Smolin 1968) do not permit examination after infliction of a stromal wound. Several other histological techniques to prepare a corneal endothelial specimen have been described by Honneger and Schierhölter (1963).
In order to prepare a flat endothelial specimen in which the presence of a stromal lesion has no disturbing effect, the cornea (with the radial incisions) is placed in its entirety between an object slide made of 5 mm thick Perspex and a cover slide of 0.4 mm thickness. Spring clamps are used to fix the cornea firmly between the two slides, with its endothelial side against the cover slide (fig. 8).
A flat endothelial specimen can thus be obtained in a manner which is simpler than the technique previously described by O'Day et al. (1973). In order to minimize displacement of the endothelial layer and consequent endothelial damage, the cornea is immersed in a 5% glutaric aldehyde solution for 15-20 seconds. To prevent adherence of the endothelial layer to the cover slide, the latter is moistened.
Good results are obtained when the radial incisions extend sufficiently far - to 2 mm from the centre. Corneas thus clamped between slides should be fixed in 5% glutaric aldehyde for at least 24 hours. This is necessary in order to ensure adequate penetration and therefore fixation of the centrally localized wound area. After these 24 hours the cover slide is removed. If no autoradiography is to be performed, nuclear staining is done, followed by dehydration in an alcohol series, clearing in xylene and embedding in Permount.

3.2.5 Autoradiography

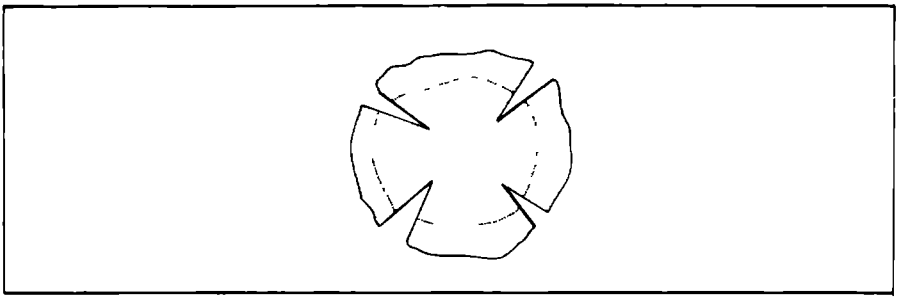
For an autoradiographic study the cornea is first dried. It is placed between two filter papers and thus placed between two objects slides. A leaden weight of 200-250 g is placed on the slides to prevent curling. In some cases the cornea nevertheless curled up afterwards. In order to minimize this, only the central 9 mm part of the cornea was used in subsequent autoradiography. The drying process takes about a day at room temperature. To ensure proper adherence of the dried cornea to the slides, the latter were gelatin-coated.
The slides were placed in a bichromate solution for 24 hours and then dipped in a 0.5% gelatin solution containing 5% chrome alum. The slides were dried in air. To fix the cornea on the slide, a droplet of heated 8% gelatin was placed on the pre-warmed gelatin-coated slide, and the cornea was placed on the droplet with the endothelial side up. To ensure good results it must be ascertained that no air is left beneath the cornea (this can be done by painting the epithelial side of the cornea with a little gelatin).



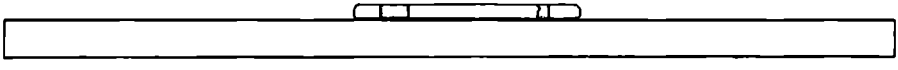
A



B



C



D

FIG. 8 *Schematic representation of the preparation of a flat corneal endothelial specimen. View from above (A) and lateral view (B) of the cornea clamped between object slide and cover slide. Perpendicular view (C) and lateral view (D) of a dehydrated cornea, fixed on an object slide and ready for autoradiography*

The cornea was then covered with a piece of Parafilm and a second slide was placed on it, whereupon a leaden weight was placed on top. The piece of Parafilm served to prevent the gelatin from adhering to the second object slide. Once the gelatin was dry, the second object glass and the Parafilm were removed. The corneal circumference was sealed all around with heated 8% gelatin in order to prevent film emulsion from creeping beneath the cornea. The specimen was then ready for autoradiography (fig. 8).
Since in this stage the cornea still tended to curl up, the interval between removal of the weight and autoradiography had to be as short as possible, preferably not more than two or three days. Before the cornea was dipped in the film emulsion (Kodak NTB, 2:1 diluted), the specimen was first moistened with distilled water. Once the specimen had been dipped in the emulsion, it was placed at an oblique angle to allow the emulsion to dry in a layer as thin as possible. The specimen was then placed in a lightproof box which contained a hygroscopic agent, and stored in the refrigerator at 4°C.
The emulsion was exposed during seven days, and then developed with Kodak D 19 film developer and fixed. Immediately after this, the nuclear stain was applied to the still wet specimen. When the background density of the film emulsion proved to be too dark due to variations in the thickness of the emulsion, making it impossible to evaluate the nuclear staining, the autoradiograph was photographically reduced and again dipped in film emulsion, dried, exposed, developed and fixed.

3.2.6 Nuclear staining

The nuclei of the endothelium in these flat specimens could best be visualized with the aid of celestine blue B, which is an oxazine dye (Gray et al. 1956). To ensure optimal staining results, the celestine blue B should be freshly prepared. The solution is obtained by adding 0.5 ml concentrated H_2SO_4 to 1 g celesine blue B and dissolving the mass thus obtained in 100 ml 2.5% chrome alum to which 14 ml glycerol has been added. The solution is then stirred thoroughly and filtered.
A staining time of 2 minutes is sufficient for a normal specimen. A specimen covered with a developed film emulsion, as after autoradiography, requires longer staining: as long as 10 minutes. Overstaining is virtually impossible. In the phase of nuclear division the entire cytoplasm is also slightly stained by the celestine blue B.
The combination of silver staining and autoradiography gave better results with celestine blue B than with haematoxylin as nuclear stain. After completion of nuclear staining the specimens were dehydrated in 95% and 100% ethylalcohol for at least 5 minutes per bath; this was followed by clearing in xylene and embedding in Permount.

3.2.7 Scanning electron microscopy

Nijmegen

The cornea was prefixed at 4°C in 2% glutaric aldehyde, 0.1 M cacodylate buffer and 4% sucrose with a total osmolality of 530 mOsm. The specimen was then placed in warm cacodylate buffer with an osmolality of 320 mOsm, with subsequent after-fixation in a 2% osmium tetroxide in phosphatic aldehyde solution.

For dehydration the specimens were passed through a series of 30%, 50%, 70%, 90% and 100% ethylalcohol and then placed in acetone for critical point dehydration with carbon dioxide. The specimen was fastened on a holder and then covered with a thin layer of gold. It was then examined in a Philips scanning electron microscope at 12 kV.

Miami

The cornea was fixed in 5% glutaric aldehyde, and the specimen was dehydrated in an ethylacohol series and acetone. Critical point dehydration was effected with carbon dioxide. The specimen was fastened on a holder and covered with a thin layer of gold. It was examined in a JEOL 35 scanning electron microscope.

3.2.8 Transmission electron microscopy

Miami

The cornea was fixed in 5% glutaric aldehyde, with after-fixation in 2% osmium tetroxide. The specimen was dehydrated in an ethylalcohol series and embedded in Epon. It was cut into sections which were studied in a JM 7 electron microscope.

RESULTS

The results obtained in this study will be presented in three sections:
In-vivo corneal endothelial wound healing in non-human primates (4.1).
In-vitro human corneal endothelial wound healing (4.2).
In-vivo human corneal endothelial wound healing (4.3).

4.1 IN-VIVO CORNEAL ENDOTHELIAL WOUND HEALING IN NON-HUMAN PRIMATES

None of the test animals developed an intraocular infection during the postoperative period. The corneas were clear from the first day on, and the eyeballs hardly injected. The animals were examined macroscopically. Occasionally, an animal was anaesthetized for slit-lamp photography, at which at the same time the cornea beyond the slit-lamp could be examined. In these cases the macroscopic findings corresponded with the biomicroscopic findings. None of the animals apppeared to be the worse for the intervention. Apart from a single application of chloramphenicol at the end of the operation, no therapy was given during the period of observation.

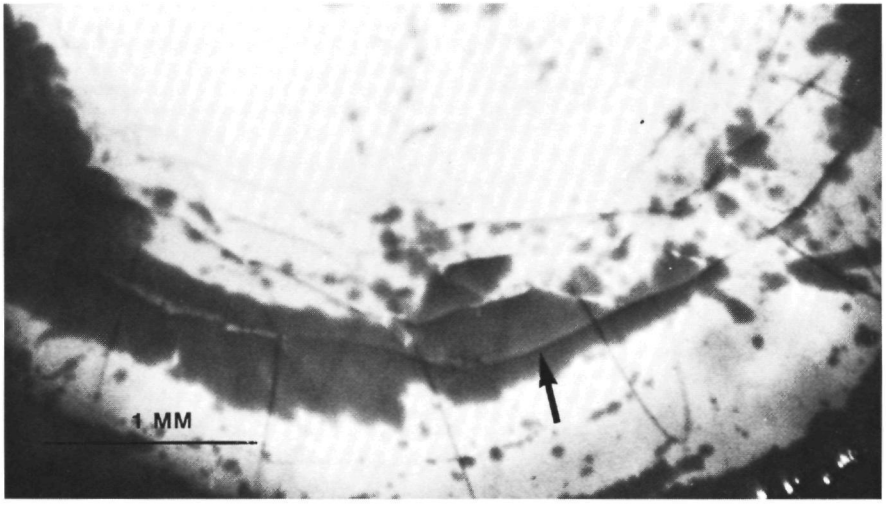


FIG. 9 *Inner corneal surface 0-3 hours after grafting. Trypan blue staining. Endothelial lesions stain with trypan blue: the dark areas in the photograph. The lesions are localized mainly along the wound margin (arrow).*

In each of the three cases it was found 0-3 hours after grafting that endothelial cell loss and damage which had occurred during the operative procedure, showed a more or less identical pattern (fig. 9).
The trypan blue-stained damaged cells and the area of Descemet's membrane which stained, were found mainly along the wound margins. The area which staind had virtually the same width all around, the peripheral margin generally showing a broader zone of damage than the central margin (fig. 9). The central part of the graft did not stain significantly with trypan blue; nor did the more peripheral part of the recipient margin.
Scanning electron microscopy (SEM) revealed a similar pattern of endothelial cell loss in these three corneas: irregularly demarcated de-endothelialized areas of Descemet's membrane localized all around along the wound margin (fig. 10). The central parts showed a normal, regular hexagonal pattern with an occasional damaged cell (fig. 10).
At some sites in the damaged endothelium the endothelial cells already showed processes indicative of incipient migrations (fig. 11).
The start of the operation can be equated to the start of endothelial damage. The interval until fixation of the cornea in glutaric aldehyde averaged 2-3 hours (this includes the time required for photography of the trypan blue-stained endothelial lesions).
The cross-sections generally showed a fairly well-adapted stromal wound. The depth of the sutures varied from two-thirds of the corneal thickness to immediately above Descemet's membrane. The central wound margin of the graft as a rule protruded further into the anterior chamber than the peripheral recipient wound margin (fig. 12).
A normal number of endothelial cells was found on Descemet's membrane in the centre. Both the central and the peripheral wound margins no longer contained any endothelial cells.
The two untreated corneas, when stained with silver, showed a normal, regular hexagonal endothelial pattern (fig. 21).

Four days after grafting:
At this time only the wound margin stained with trypan blue. The zones adjacent to the deep wound margins hardly stained any longer; nor did the centre and the periphery (fig. 13).
SEM revealed that Descemet's membrane was again covered with endothelial cells virtually as far as the deep wound margin. The cells were separated by as yet uncovered areas of Descemet's membrane. The cells presented an irregular appearance with thin processes.
The cross-sections clearly showed that in-growing epithelium filled the anterior part of the stromal wound. The graft protruded slightly further into the

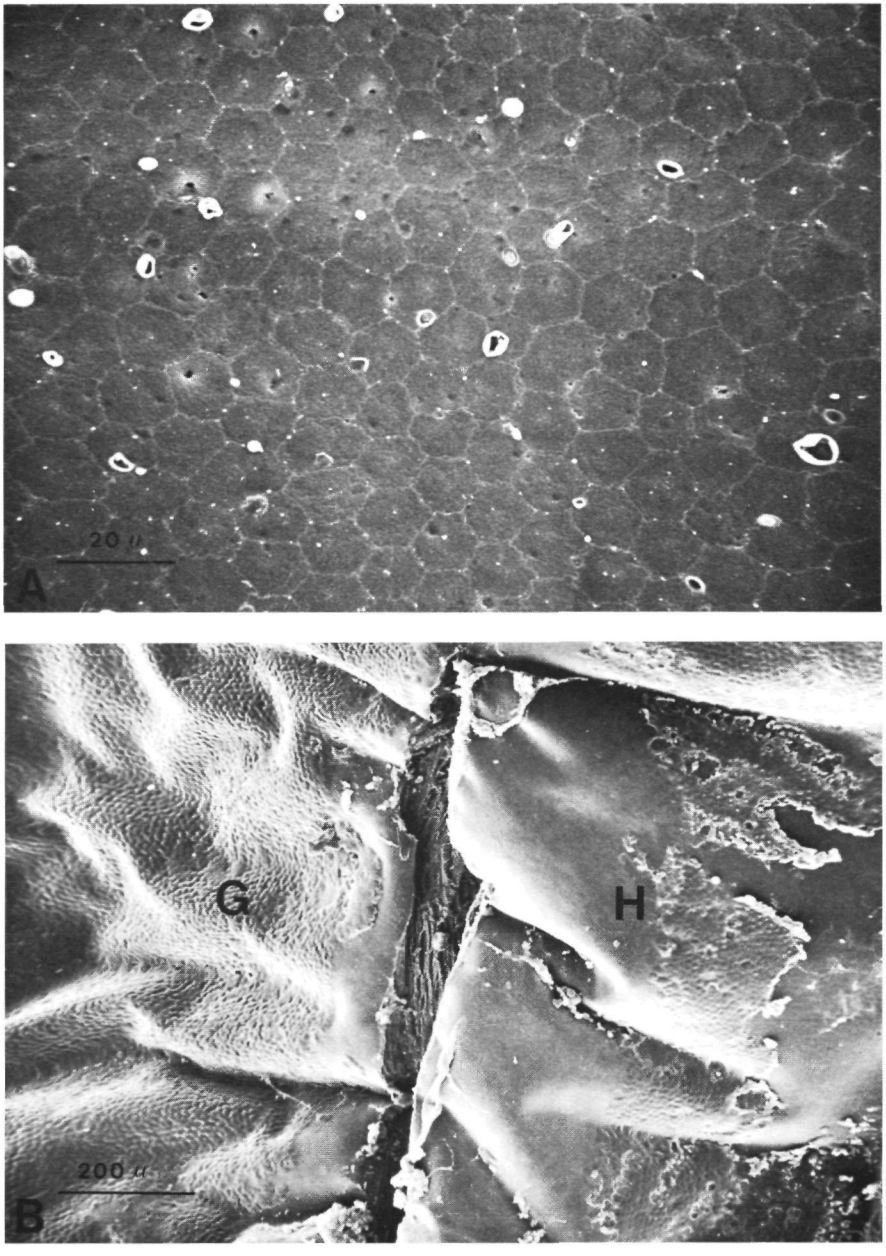


FIG. 10 *SEM of the cornea 0-3 hours after grafting, after trypan blue staining. A. The centre of the graft with a normal, regular endothelial cell pattern. B. Wound margin with endothelial loss on either side of the deep wound margin, more pronounced on the recipient side (H) than on the graft side (G).*

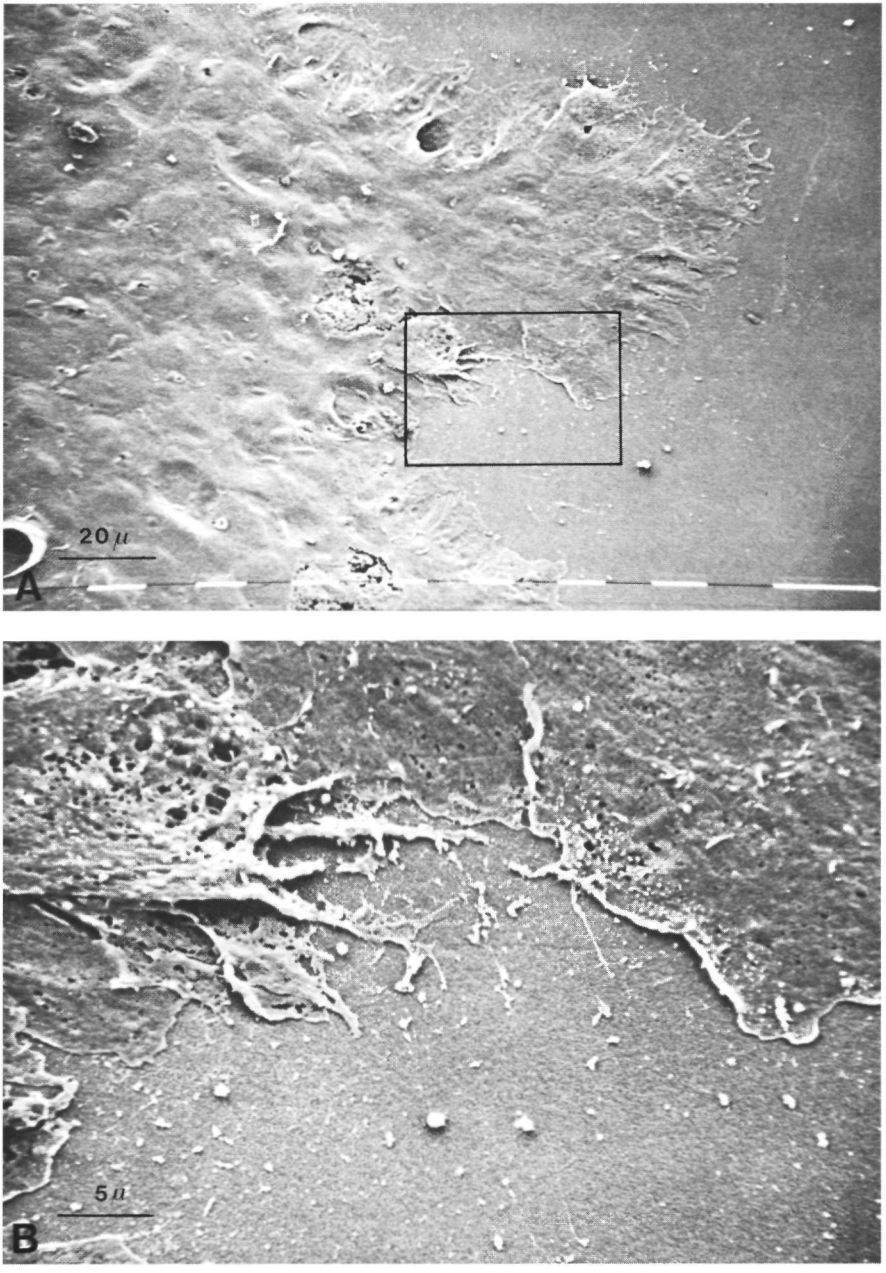


FIG. 11 *SEM of the cornea 0-3 hours after grafting, after trypan blue staining. A. start of migration of endothelial cells across an intact Descemet membrane into the damaged area. B. Higher magnification of the marked area in fig. 11A.*

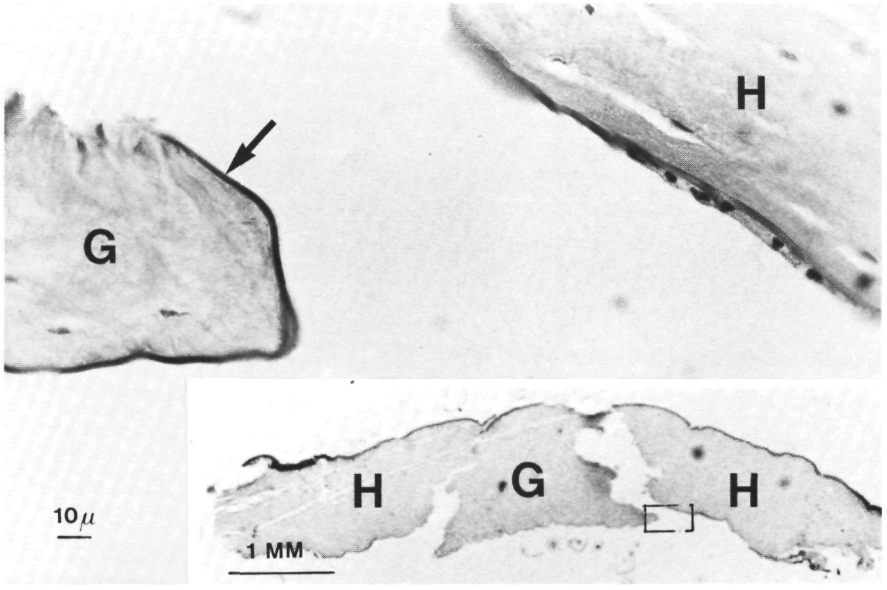


FIG. 12 *Cross-section 0-3 hours after grafting. Inset: general view of the section. Tendency of the graft (G) to override the peripheral recipient margin (H). At magnification of the area marked in the general view, few endothelial cells are visible on Descemet's membrane, which is curled in on the side of the graft (arrow).*

anterior chamber, and its wound margins slightly overlapped the peripheral wound margin (fig. 14, A and C), while on the other side of the wound the recipient margin protruded further into the anterior chamber (fig. 14, A and B). The endothelium almost completely covered Descemet's membrane as far as the deep stromal wound margin.

Eight days after grafting:
The area which stained with trypan blue was still confined to the deep wound margin proper. The central part of the graft and the peripheral margins of the recipient did not stain (fig. 15).
SEM showed well-adapted wound margins. Descemet's membrane was covered with endothelial cells virtually as far as the deep stromal wound margin. The endothelial cells were of irregular shape, and most of them had long processes. Between the cells, uncovered areas of Descemet's membrane were still visible (fig. 16).
The cross-sections showed fair adaptation of the stromal wound margins. Descemet's membrane was almost entirely covered with endothelial cells as far as the deep stromal wound margin. The deepest part of the graft protruded further into the anterior chamber. But little reaction was discernible in the wound area in the stroma proper (fig. 17).

FIG. 13 *Inner corneal surface 4 days after grafting. Only the deep stromal wound still stains with trypan blue (arrow), visible as dark areas. Note that the lesions induced during dissection of the cornea also stain with trypan blue (double arrow).*

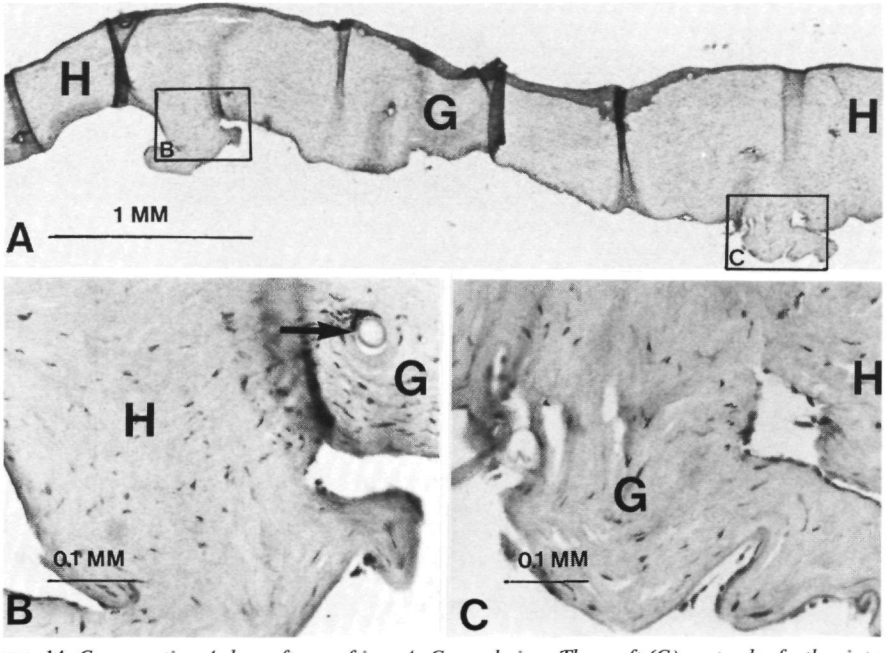


FIG. 14 *Cross-section 4 days after grafting. A. General view. The graft (G) protrudes further into the anterior chamber than the recipient margin (H) on one side of the wound (C), whereas the reverse is seen on the other side (B). At higher magnification (B and C), Descemet's membrane is seen to be covered with endothelial cells virtually as far as the wound margin. The depth at which the sutures are localized is indicated by an arrow.*

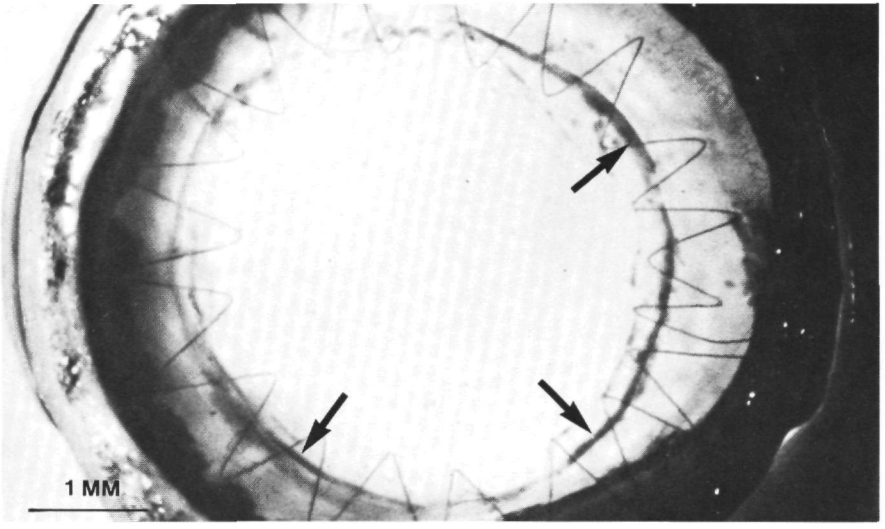


FIG. 15 *Inner corneal surface 8 days after grafting. Trypan blue staining. Only the stromal wound margin still stains with trypan blue (arrow).*

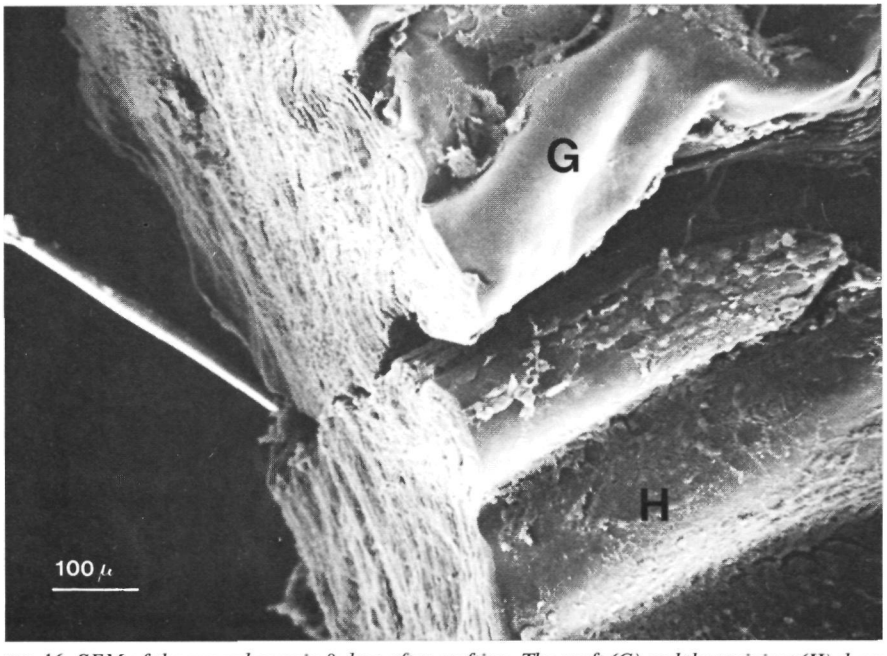


FIG. 16 *SEM of the wound margin 8 days after grafting. The graft (G) and the recipient (H) show good stromal adaptation. The graft tends slightly to overlap the recipient margin. Descemet's membrane is almost completely covered with irregular endothelial cells, some of which have long processes.*

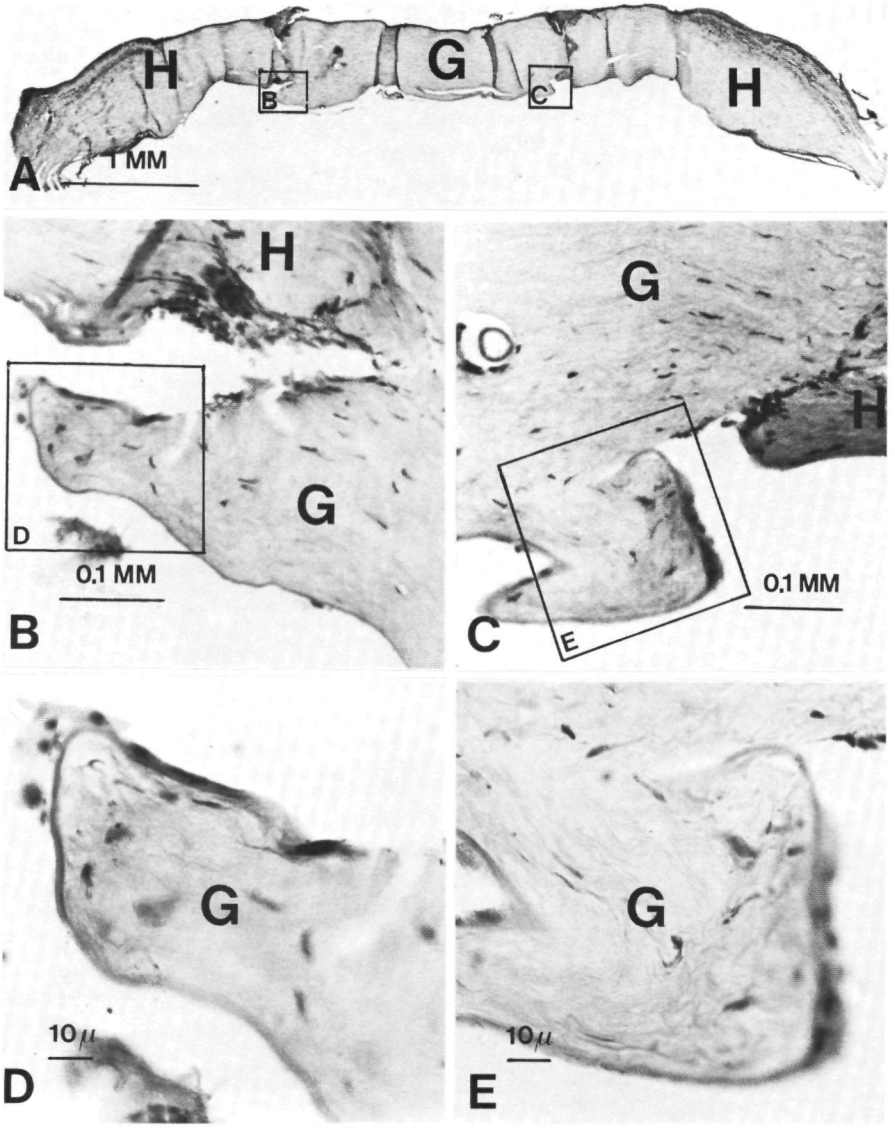


FIG. 17 *Cross-section of the cornea 8 days after grafting. A. General view of the specimen. The graft (G) protrudes further into the anterior chamber than the recipient (H). B and C are higher magnifications of the areas indicated in the general view. The stroma shows virtually no inflammatory reaction. D and E are still higher magnifications of the areas marked in B and C. Descemet's membrane is almost completely covered with endothelial cells, as far as the deep stromal wound margin.*

Twenty-eight days after grafting:
Only some parts of the deep stromal wound still stained with trypan blue (fig. 18). The centre of the graft and the peripheral margin of the recipient no longer stained.
SEM revealed a regular hexagonal endothelial cell pattern in the centre of the graft, the endothelial cells on either side of the stromal wound showing an irregular pattern (fig. 19). The fibrillar structure of the stroma was clearly visible in that part of the stromal wound that was not yet covered with endothelial cells.
The cross-sections showed a well-adapted wound on one side, where it was covered with endothelial cells. The stroma showed hardly any increase in stromal cells (fig. 20, A, B and D). An entirely different situation existed on the other side, where a mass of granulation tissue protruded from the graft into the anterior chamber. This granulation tissue showed a high degree of cellular activity.
Where this mass of granulation tissue was in contact with the peripheral part of the recipient, the stroma showed increased cellular activity (fig. 20, C). The mass itself was not covered with endothelium (fig. 20, C en D).

4.1.2 Endothelial wound healing

Regeneration of corneal endothelium after a mechanical lesion was studied.

Control:
The flat corneal specimen which was stained with silver, presented a normal hexagonal endothelial pattern. There was no marked difference between centre and periphery (fig. 21)

Day 0 after trauma:
In the silver-stained flat endothelial specimen a regular hexagonal endothelial pattern was visible, which ceased rather abruptly at the wound margin (fig. 22). Only some cellular debris was found in the wound area proper. Autoradiography revealed that tritium thymidine had neither been incorporated nor bound to the endothelial cells.

Day 1 after trauma:
The endothelial pattern was regular at the periphery. The wound margin was no longer so clearly demarcated. The wound was surrounded by a zone of transition with a width of 8-10 cells, in which the endothelial cell pattern gradually changed to large, less regular, radially arranged cells. The wound centre did not stain with silver. Autoradiography, however, showed numerous cells in this region which had incorporated tritium thymidine. Additional

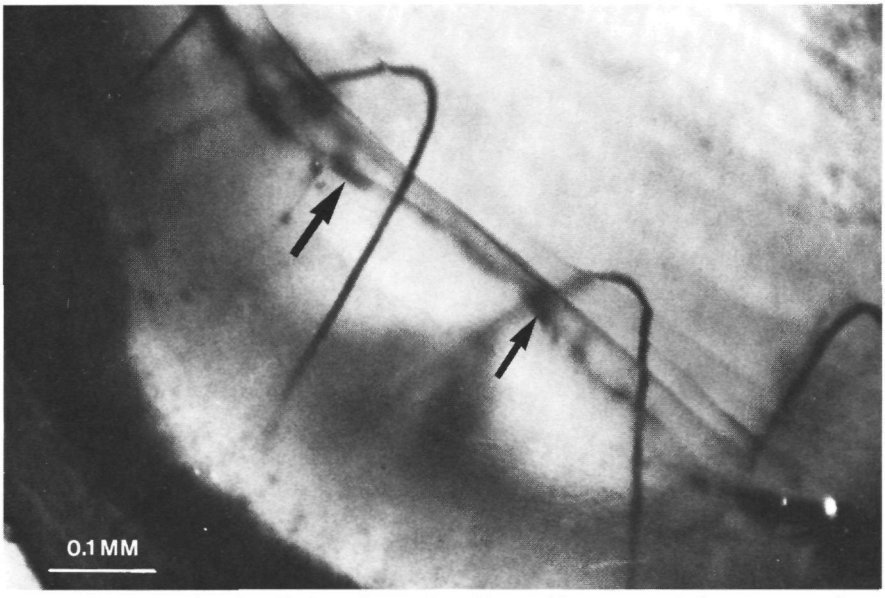


FIG. 18 *Inner corneal surface 28 days after grafting. Trypan blue staining. Only an occasional area of the deep stromal wound still stains (arrow).*

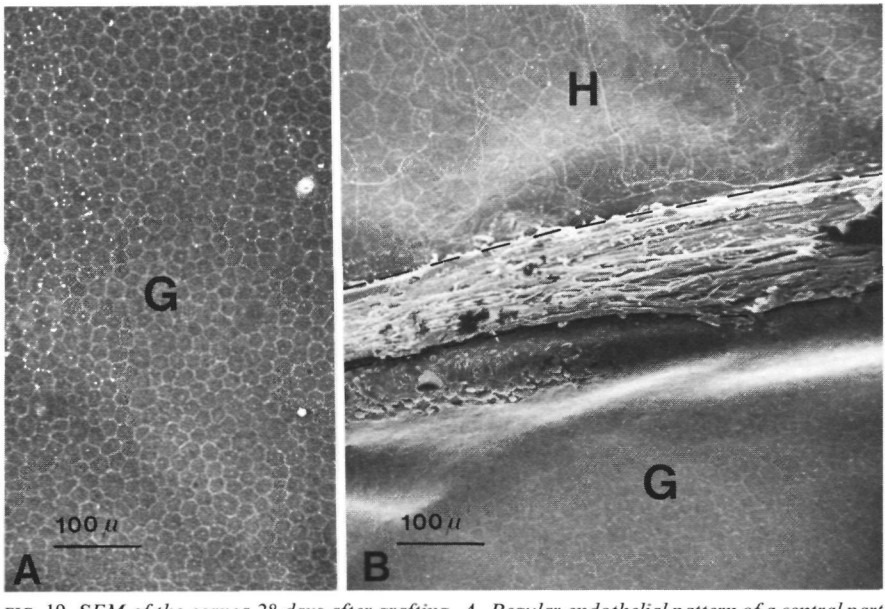


FIG. 19 *SEM of the cornea 28 days after grafting. A. Regular endothelial pattern of a central part of the graft. B. Endothelial pattern on either side of the stromal wound. Graft (G) and recipient (H). A striking feature is the difference in size of endothelial cells between the centre and the periphery of the graft. The stromal fibrils are visible within the dotted line.*

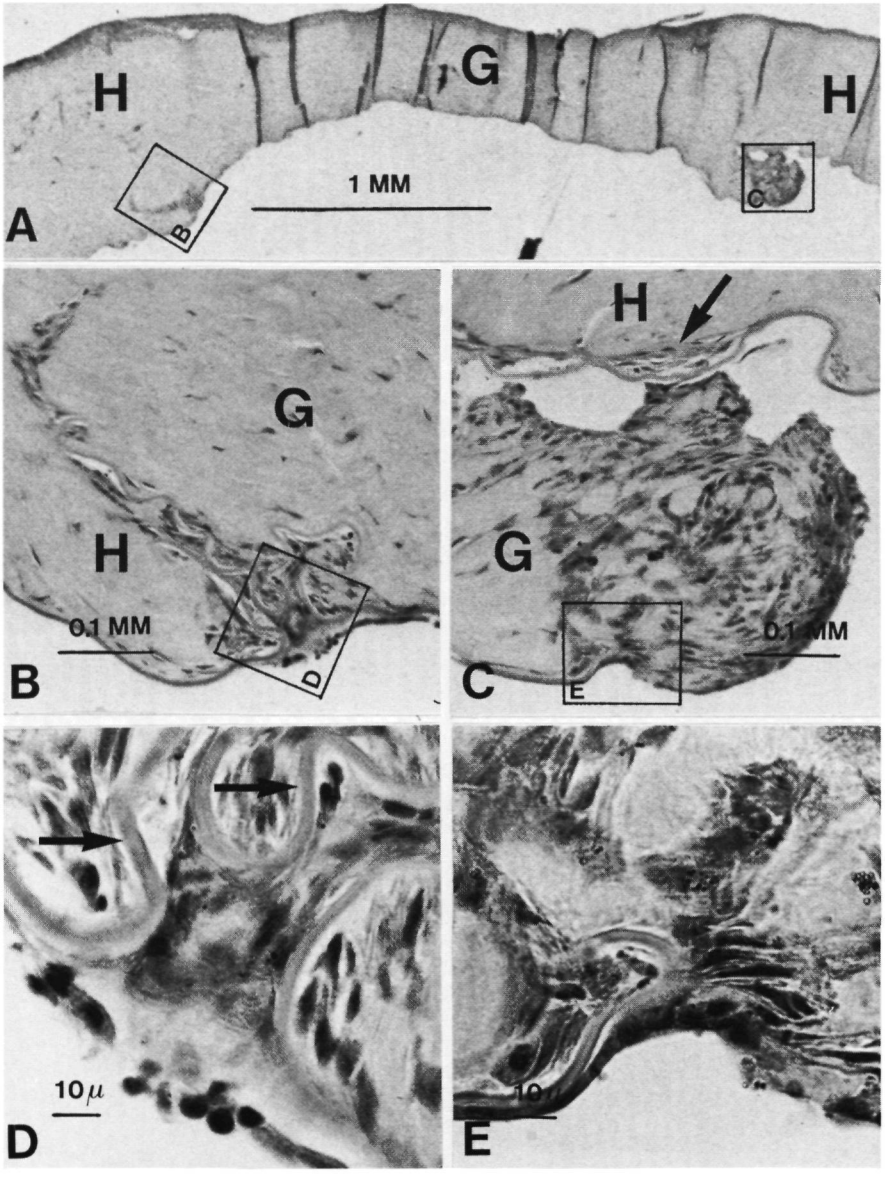


FIG. 20 *Cross-section of the cornea 28 days after grafting. A. General view. A distinct difference in adaptation between graft (G) and recipient (H). B. Well-adapted deep wound with but small cellular infiltrate. C. Granulation tissue growing from the graft stroma into the anterior chamber. Where this mass is in contact with the peripheral recipient margin, the stroma shows increased cellular activity (arrow). D. Endothelium covering the wound; the curled-in Descemet's membrane is clearly visible (arrow). E. The endothelium does not cover the mass of granulation tissue (and therefore does not cover the deep stromal wound). Increased cellular activity in the graft stroma.*

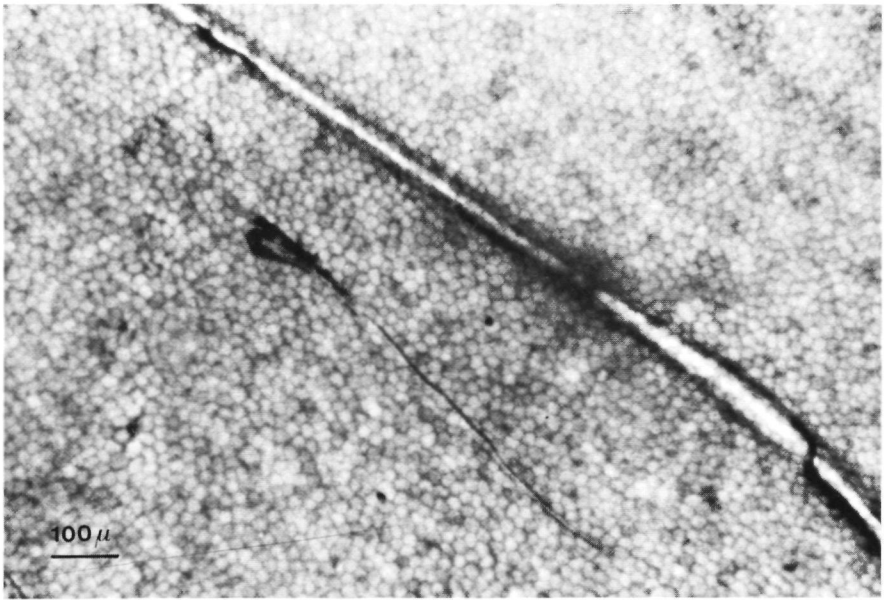


FIG. 21 *Silver-stained corneal endothelium, flat specimen. Normal hexagonal endothelial cell pattern at the centre. The corneas were originated from experiment 3.1.1.1.*

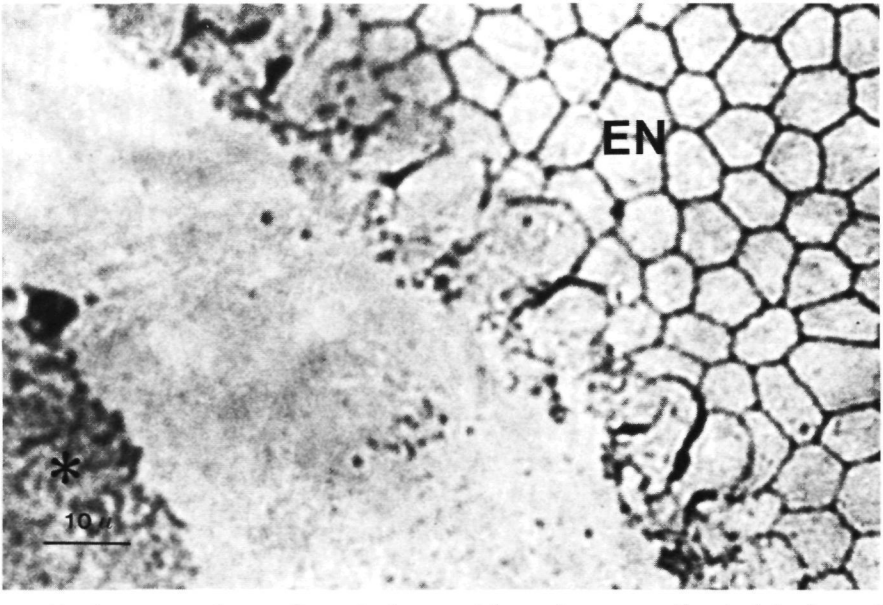


FIG. 22 *Silver stain and autoradiograph of cornea, 0 hour after trauma. Sharply defined wound margin. Normal regular endothelium is visible (EN). Cellular debris is present at the centre of the wound area (asterix).*

staining with methyl green/pyronine revealed that the wound area was populated with cells which presented a fibroblast-like appearance (fig. 23). These cells had processes which crossed each other.

Day 2 after trauma:
The cell boundaries throughout the wound now stained with silver. The endothelial cells at the periphery showed a regular pattern, while the wound proper was filled with large, irregular cells. A zone of transition separated these two cell types. The most striking feature was that in this zone of transition the cells showed an elongated radial pattern (fig. 24). Autoradiography revealed that DNA synthesis was found mainly in the wound area proper (fig. 24). One area at the periphery showed the same endothelial pattern as that at the centre. This area was the site of insertion of the lacrimal probe. A degree of endothelial damage had occurred as a result of the surgical intervention. In this area, too, a few cells had synthesized DNA.

Day 3 after trauma:
The features were virtually identical to those seen on day 2. The irregularity of the cells in the wound area seemed slightly less pronounced. The number of DNA-synthesizing cells had diminished in comparison with the number of irregular cells. Only an occasional positive cell was found in the wound area.

4.1.3 Endothelial wound healing after corneal autograft

Preparation of a flat endothelial specimen is significantly more complicated when a stromal wound is present. More artefacts occur, e.g. endothelial cell displacements and destruction of freshly formed links between acceptor and graft, particularly when, after making the radial incisions, the cornea is flattened and clamped between the slides.
However, there were enough areas without artefacts for a qualitative evaluation of the healing process; but quantitative data could not be obtained.

Hour 0-3 after autograft:
The specimen obtained immediately after completion of the surgical intervention showed large areas, on either side of the stromal wound, where Descemet's membrane was no longer lined with endothelium. All endothelial cells which stained with silver had a normal, regular cell pattern. Autoradiographic examination remained negative.

Day 1 after autograft:
The endothelial cells adjacent to the wound had arranged themselves radially in the direction of the wound. This radial arrangement was observed on either

side of the stromal wound, both on the acceptor and on the graft side. Autoradiography revealed no indication of DNA synthesis (fig. 25).

Days 2 and 3 after autograft:
There was an unmistakable increase in the irregularity of those endothelial cells that had migrated furthest into the wound area and were therefore closest to the stromal wound margin. In the zone, beside the stromal wound, the cells showed an irregular pattern; next came a zone of transition in which the radial pattern was visible, and even further away from the wound margin the cells showed a normal pattern. Autoradiography was positive both on day 2 and on day 3 after the intervention.
The cells which had incorporated tritium thymidine were found mainly among those endothelial cells that were localized closest to the stromal wound margin (figs. 26 and 27). The deep stromal wound was not covered with endothelial cells. In this wound area there were no cells that were positive in the autoradiograph.

Day 6 after autograft:
Macroscopically, the graft looked entirely clear (fig. 28).
The endothelial pattern was not essentially different from that on day 3. The

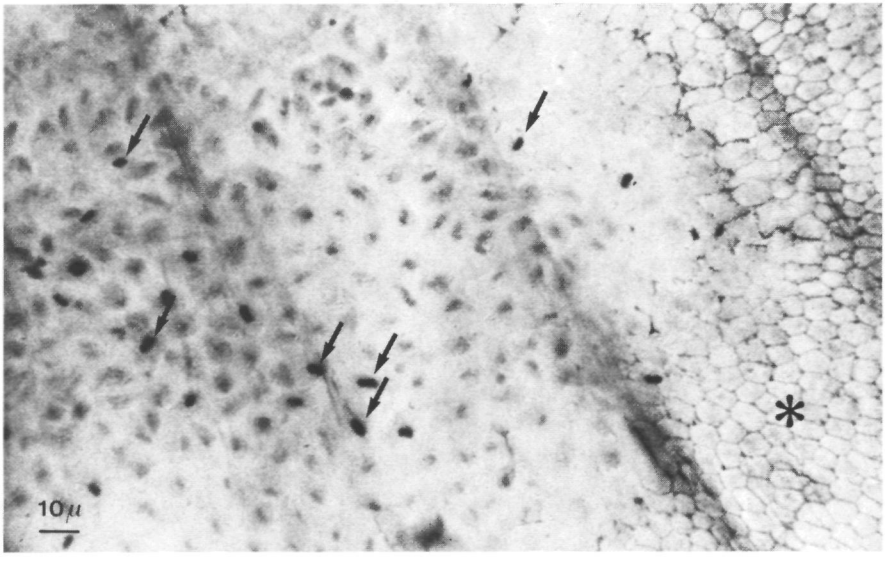


FIG. 23 *Silver stain, autoradiograph and nuclear stain of the cornea, 1 day after trauma. Silver-stained endothelial cells are radially arranged in the direction of the wound (asterix). These cells are autoradiographically negative. The wound area (left) is almost completely covered with cells that do not stain with silver. Some of these cells have incorporated tritium thymidine. A single cell of this type is marked by an arrow.*

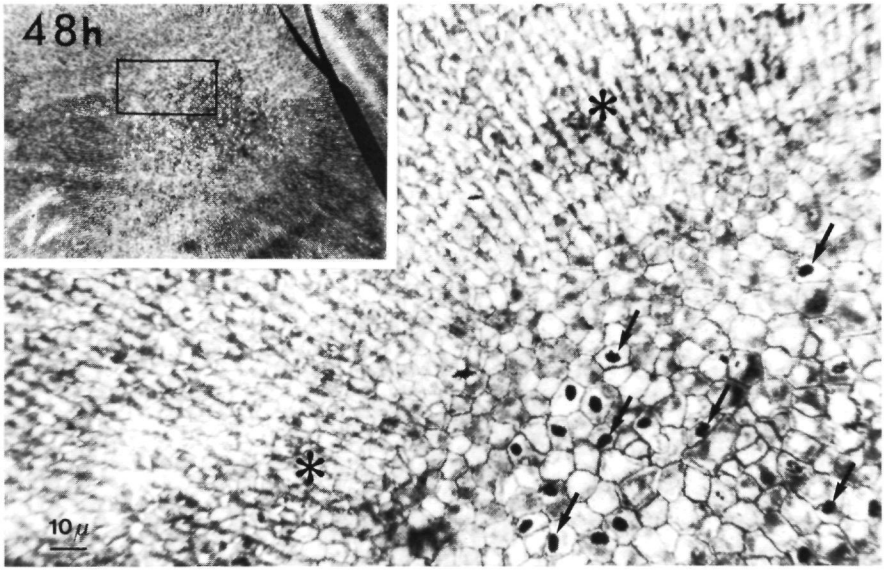


FIG. 24 *Silver stain and autoradiograph of a cornea, 2 days after trauma. The zone of transition from the regular to the irregular endothelial cell type is marked by an asterix. The wound area contains cells which have incorporated tritium thymidine (arrow). Inset: general view of the specimen.*

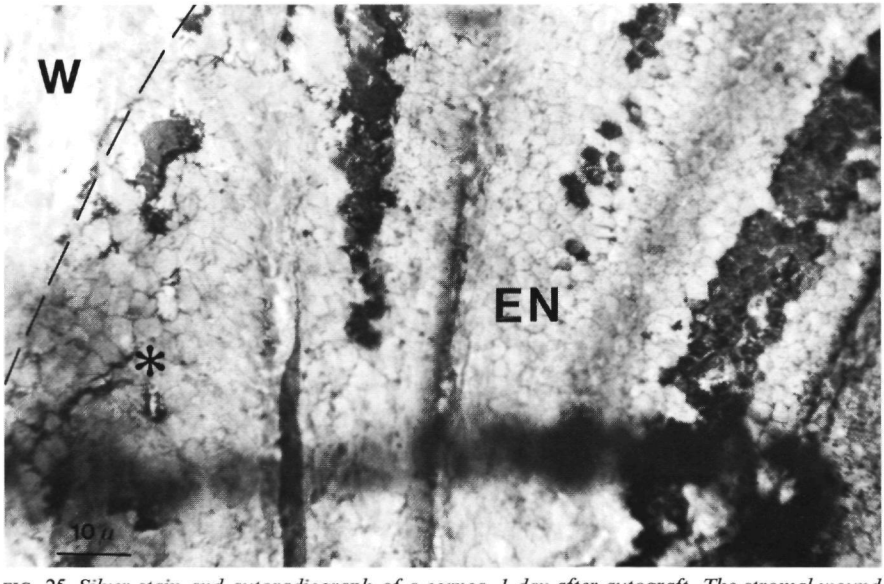


FIG. 25 *Silver stain and autoradiograph of a cornea, 1 day after autograft. The stromal wound (W) is shown top left. An irregular endothelial cell pattern along the wound margin (asterix). More centrally from the graft (right) a normal, regular endothelial cell pattern (EN) is visible. No cells are found which have incorporated tritium thymidine.*

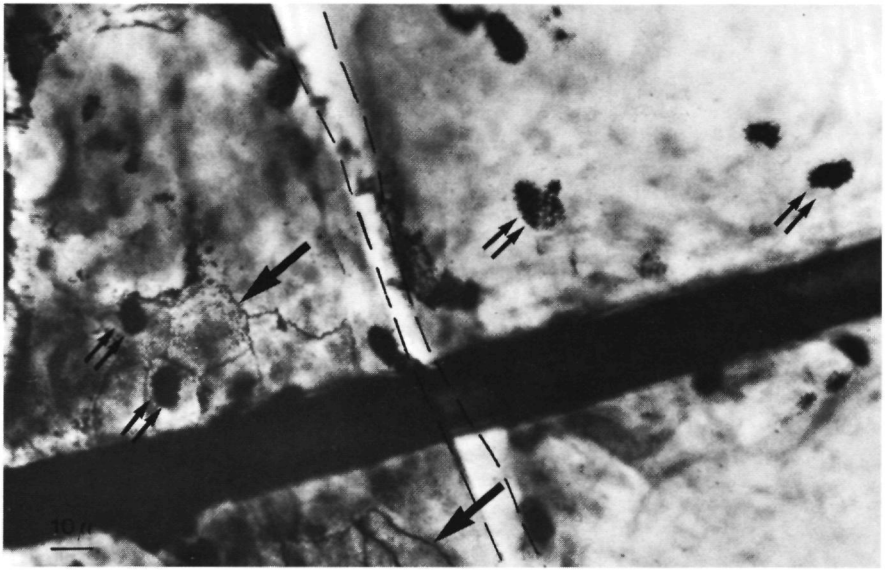


FIG. 26 *Silver stain and autoradiograph of a cornea 2 days after grafting. Irregular endothelial cells (arrow) on the recipient side of the cornea. Stromal wound marked by the dotted line. Endothelial cells which have incorporated tritium thymidine are visible on either side of the deep wound (double arrow).*

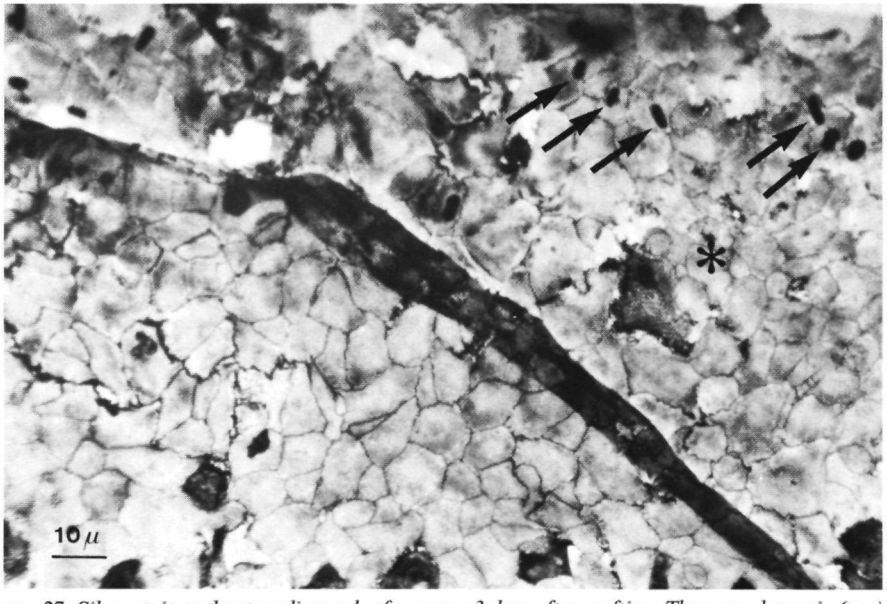


FIG. 27 *Silver stain and autoradiograph of a cornea 3 days after grafting. The wound margin (top) is covered with irregular cells (asterix), a few of which have incorporated tritium thymidine (arrow).*

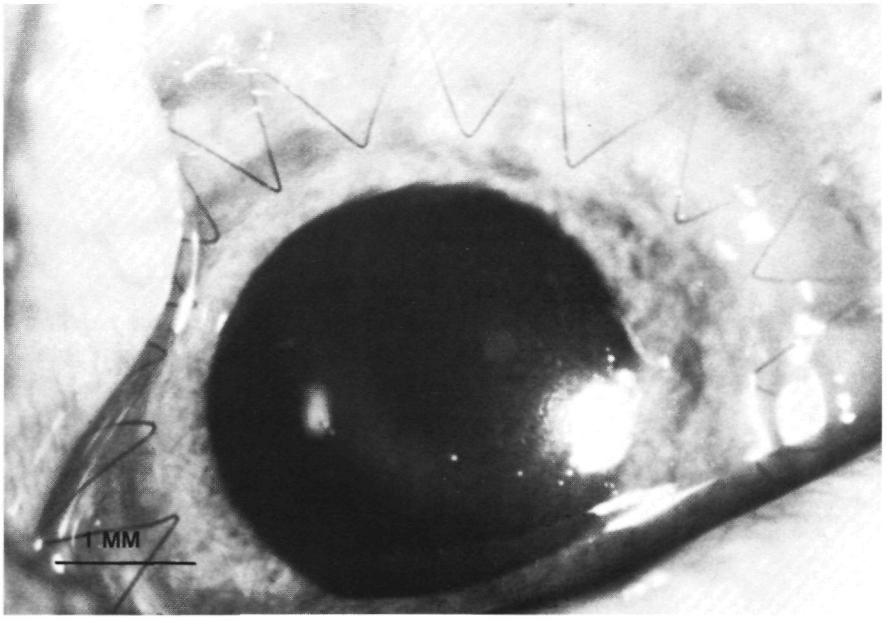


FIG. 28 *Macroscopic features of autograft 6 days after the operation. Monkey 72, OS.*

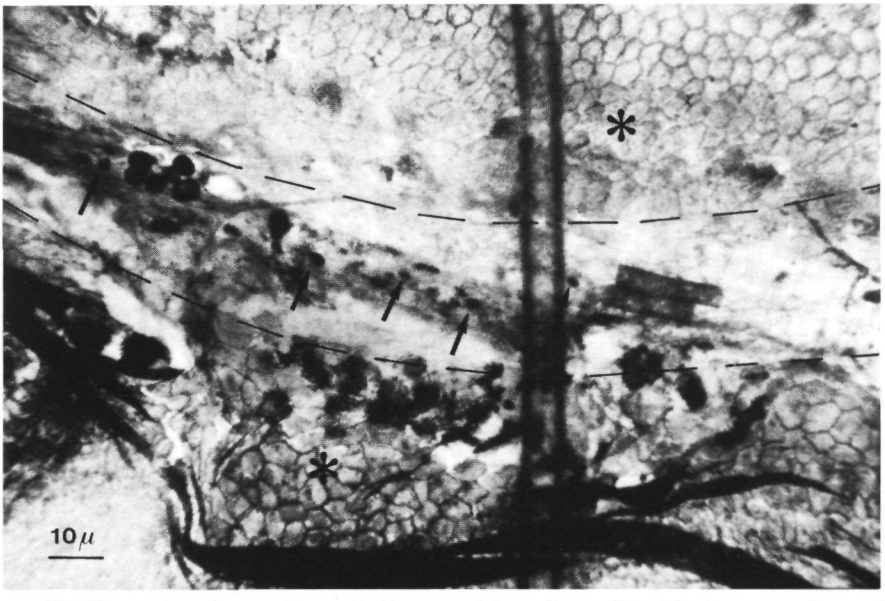


FIG. 29 *Silver stain and autoradiograph of a cornea 6 days after grafting. The deep stromal wound (within the dotted lines) is bounded on either side by irregular endothelial cells (asterix). The stromal wound contains some cells which have incorporated tritium thymidine (arrow).*

normal endothelial pattern was still clearly visible at both the centre and the periphery, the zone of transition and the irregular cells near the stromal wound margin were still in evidence (fig 29) A few of these endothelial cells were positive in the autoradiograph

The stromal wound proper did not stain with silver In this area, elongated cells were visible which were arranged more or less parallel to the longitudinal axis of the stromal wound The cells were localized in the deep wound proper, and some were positive in the autoradiograph (fig 29)

Day 13 after autograft

The endothelial pattern on either side of the stromal wound was still essentially unchanged From the irregular wound area the pattern changed via a zone of transition to a normal, regular endothelial pattern Only sporadic endothelial cells were still positive in the autoradiograph The stromal wound, however, contained longitudinally arranged cells which showed signs of DNA synthesis (figs 30 and 31)

4 2 IN-VITRO HUMAN CORNEAL ENDOTHELIAL WOUND HEALING

The results obtained by studying wound healing in cultured human corneas will be discussed per experiment

4 2 1 Standardization of endothelial wounds

The purpose of this experiment was to establish whether a standard size corneal endothelial wound could be obtained with the woundinflicting apparatus described After infliction of a wound, 14 corneas were immediately stained with silver and fixed In all cases the wound margin was well-defined The normal hexagonal endothelial pattern was clearly visible immediately

TABLE 4 2 1 *Wound dimensions in silver stained corneas with a standard mechanical endothelial wound*

Cornea	HD	VD	Cornea	HD	VD
1	2 36	2 25	8	2 38	2 30
2	2 36	2 29	9	2 37	2 29
3	2 38	2 27	10	2 36	2 29
4	2 36	2 29	11	2 36	2 30
5	2 35	2 30	12	2 38	2 29
6	2 37	2 28	13	2 38	2 27
7	2 36	2 29	14	2 35	2 30

HD = horizontal diameter in mm VD = vertical diameter in mm

FIG. 30 *Silver stain and autoradiograph of a cornea 13 days after grafting. An irregular endothelial cell pattern (asterix) is visible on either side of the stromal wound (within the dotted lines). The endothelium of the graft (top right) is partly displaced as a result of processing. Autoradiograpically positive cells (arrow) are visible in the stromal wound (see also fig. 31).*

FIG. 31 *Silver stain and autoradiograph of a cornea 13 days after grafting. Magnified detail of fig. 30. A few irregular endothelial cells are visible (asterix). Cells which have incorporated tritium thymidine (arrow) are visible; they parallel the longitudinal axis of the stromal wound.*

outside the wound area. Both the horizontal and the vertical diameter of the wound were measured with the aid of a microscopic gradation. The measurements were obtained from the fixed flat corneal specimen (table 4.2.1; fig. 32). The mean horizontal wound diameter was 2.37 mm (SD 0.01 mm). The mean vertical wound diameter was 2.286 mm (SD 0.015 mm). The total wound surface area was about 4.2 mm^2. In none of the specimens a rupture of Descemet's membrane was observed.

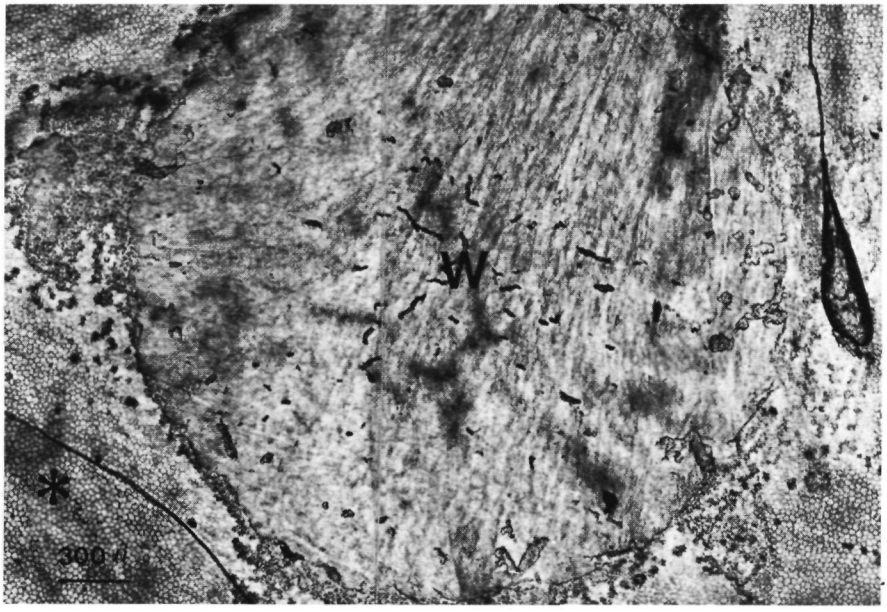


FIG. 32 *Silver stain of a cornea with a standard wound (W). Only cellular debris is found in the wound area proper, while outside the wound the normal endothelial cell pattern is clearly visible (asterix).*

4.2.2. Control

Of the 19 pairs of corneas available for experiments 4.2.2. and 4.2.3, one cornea of a pair was sometimes used in experiment 4.2.2, while the other cornea was used in experiment 4.2.3.
This experiment (11 corneas) served as control for subsequent experiments (survey A).
The corneas were cultured for 2, 3 or 4 days, tritium thymidine being added to the medium (5 μCi/ml) at the start of the culture period. A normal, regular endothelial pattern was observed at the centre of each of the 11 corneas (fig. 33).

SURVEY A

Survey of 11 corneas. Continuous exposure to tritium thymidine. Standard wound.

Donor age in years	Duration of culture		
	2 days	3 days	4 days
0- 9			
10-19			
20-29			●●●
30-39			
40-49	●●	●	
50-59	●●●		
60-69			●
70-79	●		
80-89			
Number of APC in the wound	0	0	0
Average number of APC	0	0	0

APC = autoradiographically positive cells.
● = autoradiographically negative specimen.

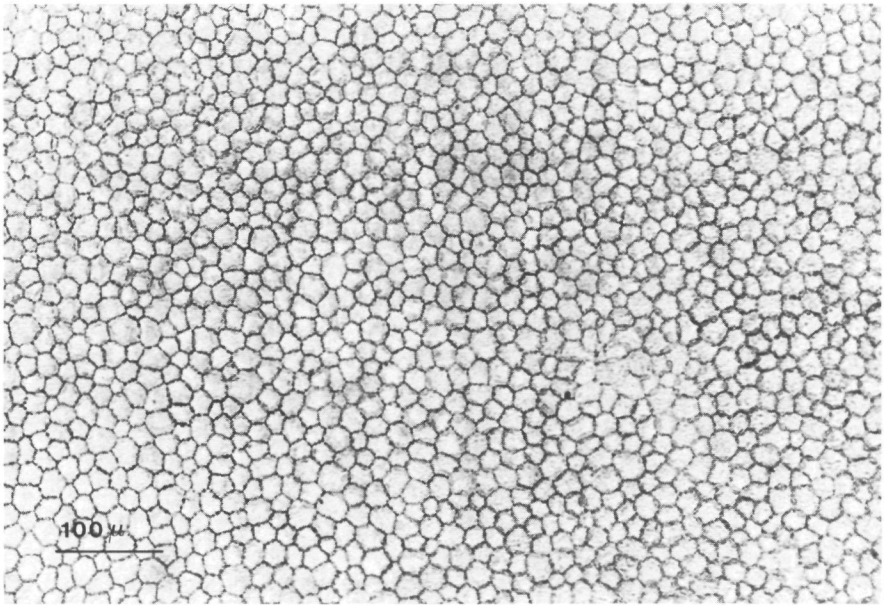


FIG. 33 *Silver stain and autoradiograph of a cornea cultured for 4 days. Normal regular endothelial cell pattern without signs of incorporation of tritium thymidine. Donor aged 29. Compare these endothelial features with those in the monkey in fig. 21.*

In none of the corneas incorporation of tritium thymidine was observed at the corneal centre. At the periphery, however, sometimes solitary, sometimes groups of cells were found which had incorporated tritium thymidine. In none of the specimens a mitotic figure was observed (table 4.2.2).

TABLE 4.2.2 *Autoradiographic results in 11 cultured human corneas. Duration of culture 2, 3 or 4 days. Continuous exposure to tritium thymidine. No endothelial wound*

Pair of corneas number	Age in years	Duration of culture 2 days	3 days	4 days	Donor time* in hours
1	61			neg	13
2	22			neg	14
3	51	neg			9
4	73	neg			15
5	29			neg.	17
5**	29			neg.	17
6	46		neg.		24
7	45	neg.			20
7**	45	neg.			20
8	53	neg.			14
8**	53	neg.			14

* Donor time = interval between death and start of culture.
** Second cornea of a pair. The pairs of corneas of experiments 4.2.2 and 4.2.3 are numbered consecutively.

4.2.3 DNA synthesis?

The question whether DNA synthesis is a feature of endothelial wound healing was considered. The purpose of this experiment was to establish whether wound healing occurred and whether it involved only migration or also proliferation of endothelial cells.
The corneas used were cultured for 2, 3 or 4 days, tritium thymidine being added to the culture medium (5 μCi/ml) at the start of the culture.
In this group of 20 corneas a normal endothelial cell pattern was found only in the more peripheral parts of the cornea. In the wound area the endothelial cells showed an irregular pattern. The wound area was surrounded by a zone of transition in which this cell pattern gradually changed to a normal cell pattern (fig. 34).
The wound area proper showed a gradual transition from areas with silver-staining cells to areas in which cells were present but did not (yet) stain with silver (fig. 35).
After two days, two of the eight corneas showed silver staining of the cells at the centre; after three and four days all cells in the wound area stained with silver.

SURVEY B

Survey of 20 corneas. Continuous exposure to tritium thymidine. Standard wound.

Donor age in years	Duration of culture		
	2 days	3 days	4 days
0- 9		○	
10-19	○		
20-29		○	○○
30-39	○○		○
40-49			
50-59	○○		○○
60-69	○○	○	○○
70-79	○	○○●	
80-89			
Number of APC in the wound	2-924	0-392	176-2283
Average number of APC	147	71	1271

APC = autoradiographically positive cells.
● = autoradiographically negative specimen.
○ = autoradiographically positive specimen.

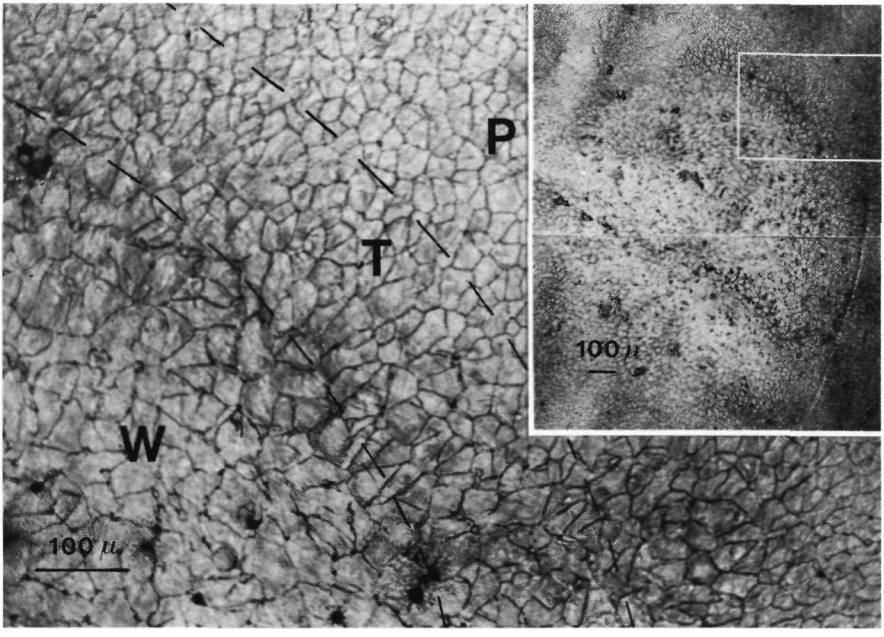


FIG. 34 *Silver stain of human cornea cultured for 3 days. Inset: general view of the wound area. The area shown at higher magnification is encircled. The cells in the wound area (W) present an irregular appearance, whereas those at the periphery (P) show a normal endothelial pattern. A zone of transition (T) is seen in between. Donor aged 30.*

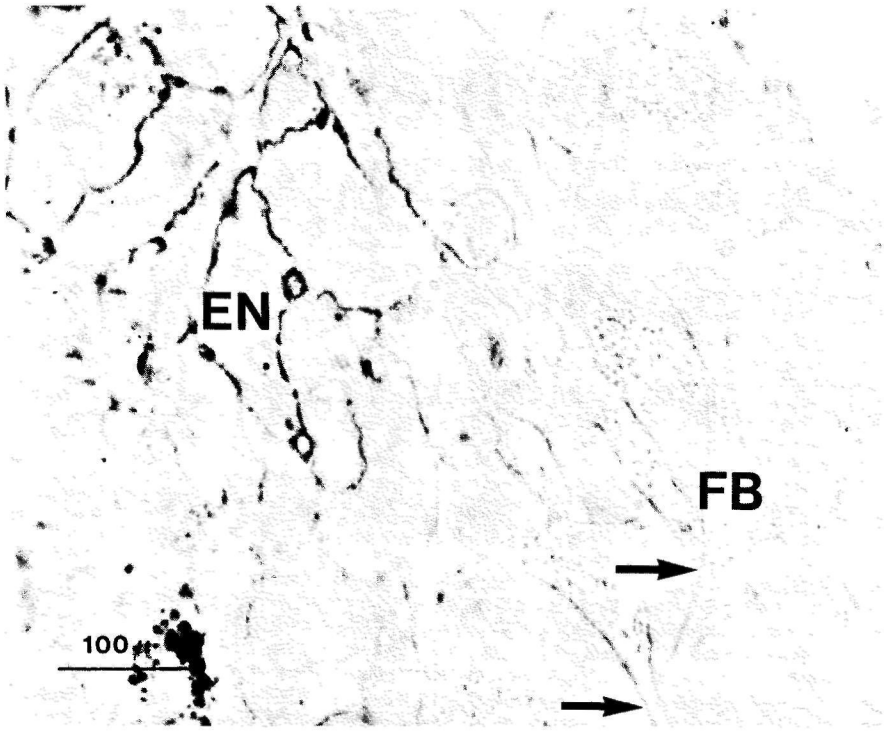


FIG. 35 *Silver stain of human cornea cultured for 2 days. Gradual change of cells which populate the wound area and stain with silver. On the right, endothelial cells which still show fibroblast-like features (FB). The processes of these cells cross each other (arrow). These cells do not (yet) stain with silver. On the left, endothelial cells which do stain with silver. These cells present an irregular appearance (EN). Donor aged 38.*

In 19 of the 20 corneas, the cells in the central wound area had incorporated tritium thymidine (see table 4.2.3, survey B). The difference between silver-stained corneas before and after autoradiography was quite unmistakable (fig. 36).

Autoradiography revealed sometimes solitary, sometimes groups of DNA-synthesizing cells at the periphery of some specimens. Nuclear staining showed that the nuclei of the endothelial cells in the wound area and in the zone of transition were larger than those of more peripherally localized endothelial cells. In none of the specimens a mitotic figure was observed.

There was no autoradiographically demonstrable difference between the 13 corneas placed in a culture medium containing unlabelled thymidine at the end of the experiment, and those not so treated.

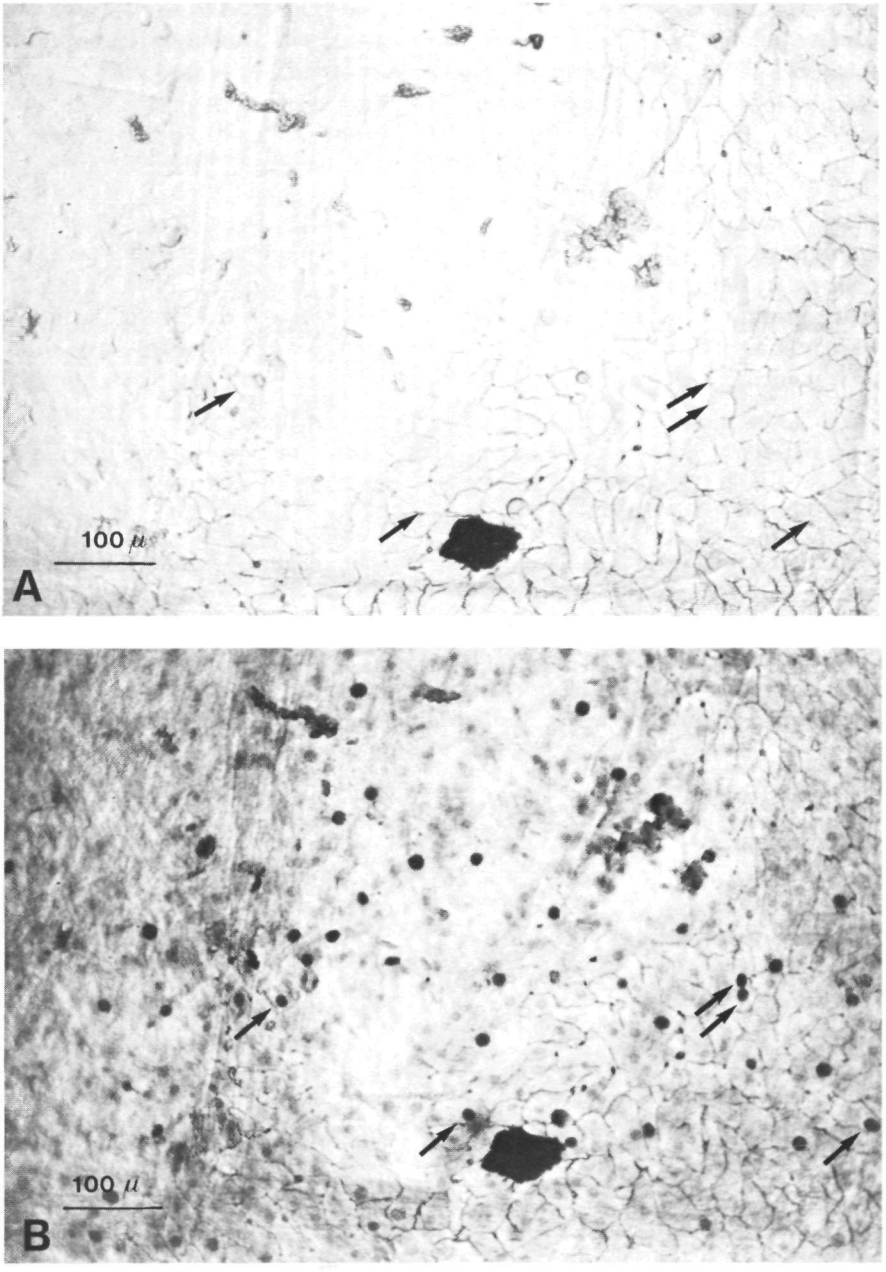


FIG. 36 *Silver stain and autoradiograph of human cornea cultured for 2 days. The same area shown before (A) and after (B) autoradiography. Cells whose nuclei have incorporated tritium thymidine are marked by arrows Donor aged 51.*

TABLE 4.2.3 *Autoradiographic results in 20 cultured human corneas. Duration of culture 2, 3 or 4 days. Continuous exposure to tritium thymidine. Standard endothelial wound. Figures in brackets indicate numbers of labelled nuclei in the wound area.*

Pair of corneas number	Age in years	Duration of culture 2 days	3 days	4 days	Donor time* in hours	Wound closed
1**	61			pos. (176)	13	yes
2**	22			pos. (1283)	14	yes
3**	51	pos. (76)			9	no
4**	73	pos. (2)			15	no
9	19	pos. (924)			14	yes
10	71		neg.		19	yes
10**	71		pos. (2)		19	yes
11	76		pos. (5)		18	yes
12	38	pos. (8)			15	no
12**	38	pos. (10)			15	yes
13	61		pos. (392)		19	yes
14	51	pos. (135)			17	no
15	64	pos. (31)			19	no
15**	64	pos. (12)			19	no
16	1		pos. (12)		4	yes
17	30		pos. (12)		16	yes
17**	30			pos. (2283)	16	yes
18	25			pos. (1976)	10	yes
19	52			pos. (773)	17	yes
19**	52			pos. (1136)	17	yes

* Donor time = interval between death and start of culture.
** Second cornea of a pair. The pairs of corneas of experiments 4.2.2 and 4.2.3 are numbered consecutively.

4.2.4 DNA synthesis and/or mitosis

In this experiment, DNA synthesis was studied in relation to the interval since infliction of a standard wound. Mitotic figures were looked for as well. The corneas were cultured for 1, 2, 3 or 4 days. Tritium thymidine was added to the culture medium (5 μCi/ml) one hour before completion of the experiment so that incorporation of thymidine could take place only during this hour. The total number of pairs of corneas available for this experiment was 26.

Day 1 after trauma in vitro:
The silver staining of the specimens revealed that the endothelial cells had migrated into the wound. In the most central area these cells presented an irregular appearance; next came a zone of transition in which the cells gradually changed to the normal cell pattern.
The wound surface area of a few wounds was measured on day 1. As compared with the surface area of the standard wound, the wound area which failed to stain with silver proved to be reduced by about 50% on day 1. This was entirely

Survey of 44 corneas Pulsed exposure to tritium thymidine. Standard wound

Donor age in years	Duration of culture			
	1 day	2 days	3 days	4 days
0- 9			○○	
10-19			○○○○○	
20-29		○●	○●	
30-39		○○○○	○○	
40-49	●●●●	○○○○	○○	○●●
50-59	●●	○	○	
60-69	●	○	○	○○○●●
70-79	●			●
80-89				
Number of APC in the wound	0	0-292	0-220	0-31
Average number of APC	0	126	61	9

APC = autoradiographically positive cells
● = autoradiographically negative specimen.
○ = autoradiographically positive specimen.

a result of the migration process. No difference in pattern and rate of migration was found between corneas of different age (fig. 37). Wound dimensions were not systematically measured on day 1.
Autoradiography was negative with all 24-hour specimens. Nuclear staining disclosed that the nuclei of the migrated cells were larger than those of the cells at the periphery, which showed no sign of migratory activity.

Day 2 after trauma in vitro:
Examination of the silver-stained specimens revealed that the wound area was closed in 4 of the 12 cases. The cell pattern was not essentially different from that in the 24-hour specimens. The endothelium showed a zone of transition at the wound margin. The more centrally localized cells showed an irregular pattern, whereas those at the periphery were normal in this respect.
Autoradiography was positive with 11 of the 12 corneas. The labelled nuclei were exclusively found in the wound area. The number of positive nuclei per wound varied rather widely (see table 4.2.4, survey C). The number of positive labelled nuclei per wound averaged 126. A few positive nuclei were found at the periphery, sometimes in small groups, sometimes solitary.
Nuclear staining revealed mitotic figures in two corneas. Metaphases were involved in both. The nuclei of the transformed cells were larger than those of the normal endothelial cells at the periphery.

Day 3 after trauma in vitro:
Silver staining showed that three of the 15 corneal wounds had not yet closed.

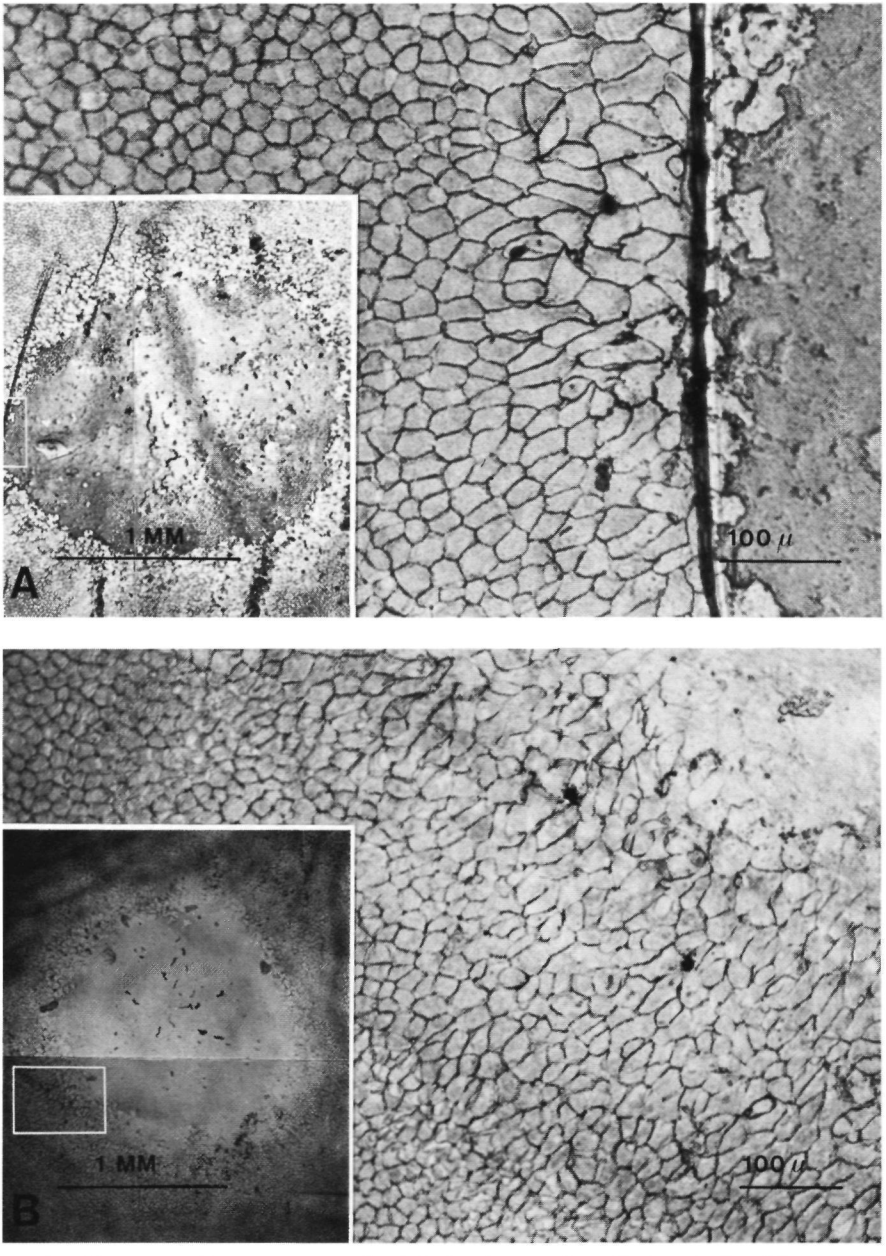


FIG. 37 *Silver stain of human corneas after 1 day's culture in vitro. The inset in A and B presents a general view of the endothelial wound. The area shown at higher magnification is demarcated. A. Cornea of a 13-year-old donor (cultured in a preliminary experiment). B. Cornea of a 71-year-old donor.*

TABLE 4 2 4 Autoradiographic results in 44 cultured human corneas Duration of culture 1, 2 3 or 4 days Pulsed exposure to tritium thymidine Standard endothelial wound Figures in brackets indicate numbers of labelled nuclei in the wound area

Pair of corneas number	Age in years	Duration of culture 1 day	2 days	3 days	4 days	Donor time* in hours	Wound closed
1	60				neg	70 MK	yes
2	71	neg				29 MK	no
2**	71				neg	29 MK	yes
3	35			pos (63)		25 MK	yes
4	45			pos (4)		15	yes
4**	45				pos (17)	15	yes
5	52		pos (39)			13	no
5**	52			pos (5)		13	no
6	37		pos (196)			15	no
6**	37			pos (120)		15	no
7	65		pos (220)			15	no
7**	65	neg				15	no
8	40	neg				12	no
8**	40		pos (38)			12	yes
9	41		pos (8)			12	yes
9**	41			pos (25)		12	yes
10	23		neg			12	no
11	27		pos (142)			12	yes
12	30		pos (78)			18	no
13	49	neg				17	no
14	66			pos (17)		18	yes
15	16			pos (220)		9	no
16	16			pos (23)		15	yes
16**	16			pos (90)		15	yes
17	35		pos (212)			15	no
17**	35		pos (292)			15	no
18	69				pos (30)	18	yes
18**	69				pos (6)	18	yes
19	45		pos (150)			18	no
19**	45		pos (120)			18	yes
20	67				pos (30)	17	yes
20**	67				neg	17	yes
21	45	neg				19	no
21**	45	neg				19	no
22	25			pos (95)		11	yes
22**	25			neg		11	yes
23	2			pos (30)		17	yes
23**	2			pos (45)		17	yes
24	45				neg	13	yes
24**	45				neg	13	yes
25	50	neg				13	no
25**	50	neg				13	no
26	15			pos (180)		11	yes

* Donor time = interval between death and start of culture
** Second cornea of a pair
MK = Cornea stored in McCarey Kaufman Medium at 4°C

The cell pattern was similar to that in 48-hour specimens. Autoradiography was positive with 14 of the 15 corneas (table 4.2.4). In this group, too, the number of positive labelled nuclei per wound varied, the average number of labelled nuclei per standard wound now being 61. In none of the specimens DNA synthesizing cells were found at the periphery.
In this group the largest number of mitotic figures was found. A few mitotic figures were found in five of the 15 corneas (fig. 38).

Day 4 after trauma in vitro:
On day 4, all wounds had closed. The cell pattern was again irregular in the wound area, although differences seemed less pronounced. Autoradiography was positive with four of the nine corneas. The number of positive labelled nuclei per wound was smaller and varied less widely (table 4.2.4). The number of labelled nuclei per wound averaged 9.
In some cases nuclear staining could not be evaluated because the background density of the autoradiograph was too intensive.
The number of positive labelled nuclei per standard wound diminished with a

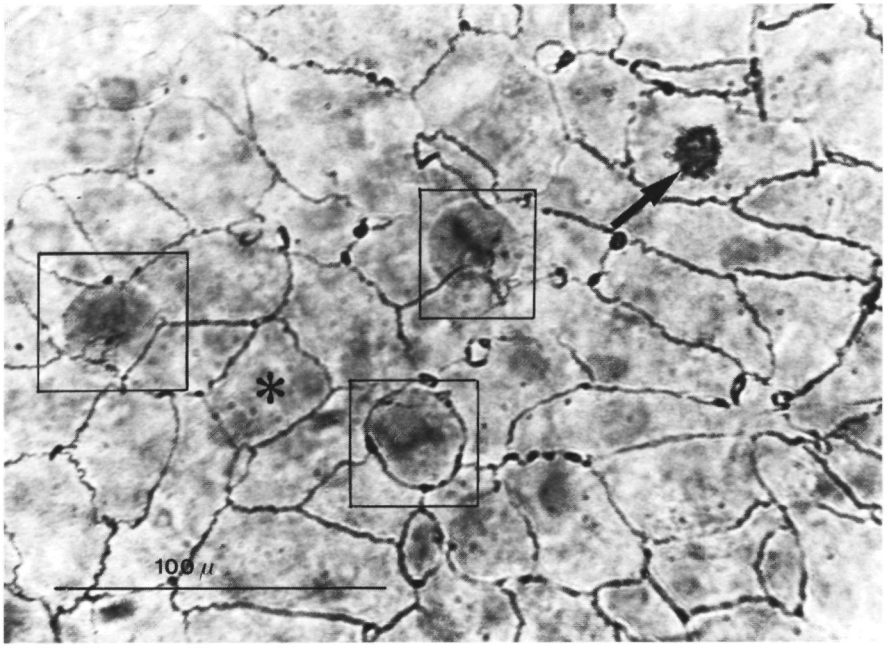


FIG. 38 *Silver stain, autoradiograph and nuclear stain of a human cornea cultured for 3 days. Part of the central wound area. Silver staining shows the irregular cell pattern (asterix); autoradiography reveals the presence of DNA-synthesizing cell (arrow); mitotic figures (metaphasic) are encircled Donor aged 41.*

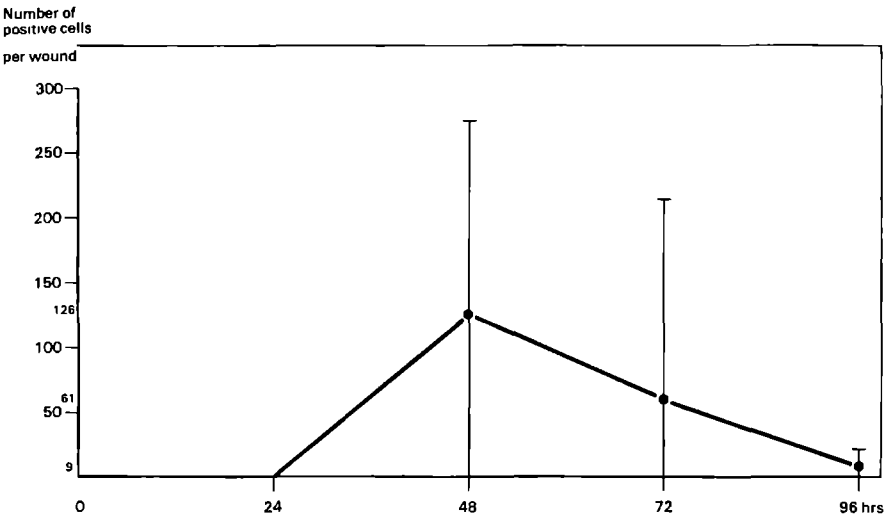


FIG. 39 *Number of positive labelled nuclei of endothelial cells per standard wound (average), in relation to the duration of corneal culture.*

longer duration of culture and a longer period in which the endothelial wound had an opportunity to close (fig. 39).
The pattern of closing of corneal endothelial wounds in vitro is shown in figs. 40 and 41.

4.2.5 Autoradiographically positive mitotic figure

The purpose of this experiment was to find autoradiographically positive mitotic figures. All corneas were cultured for 3 days, tritium thymidine being added to the culture medium (1 μCi/ml) at the start of the experiment. Five pairs of corneas were available. Silver staining was omitted.
After development of the autoradiograph it was found that in all cases the background density was such that, although it was possible to establish whether the nuclei had incorporated tritium thymidine, evaluation of nuclear staining was impossible. This prompted the decision to reduce photographically.
All specimens were again dipped in film emulsion. After development of the second film coating the quality of alle ten specimens proved to be perfect. Nuclear staining was then done immediately.
DNA-synthesizing cells were present in all corneas, synthesis again being confined to the wound area proper. The number of labelled nuclei per wound averaged 900 (table 4.2.5, survey D).

Survey of 10 corneas. Continuous exposure to tritium thymidine. Standard wound

Donor age in years	Duration of culture 3 days
0- 9	OO
10-19	OO
20-29	OO
30-39	
40-49	OOOO
50-59	
60-69	
70-79	
80-89	
Number of APC in the wound	452-1442
Average number of APC	900

APC = autoradiographically positive cells
• = autoradiographically negative specimen
O = autoradiographically positive specimen

Tritium thymidine mitotic figures were found in five of the specimens (fig. 42). No autoradiographically negative mitotic figures were found. Despite the much lower thymidine concentration (1 μCi/ml) than in the comparable experiment 4.2.3 (5 μCi/ml), the density of the autoradioraph was often still too intensive to permit proper evaluation of the underlying nuclei with nuclear staining (fig. 42).

TABLE 4 2.5 *Autoradiographic results in 10 cultured human corneas. Duration of culture 3 days Continuous exposure to tritium thymidine. Standard endothelial wound. Figures in brackets indicate numbers of labelled nuclei in the wound area.*

Pair of corneas number	Age in years	Three days' culture	Donor time* in hours	Number of labelled mitotic figures
1	49	pos. (490)	19	0
1**	49	pos. (452)	19	0
2	25	pos (1442)	9	0
2**	25	pos (658)	9	1
3	49	pos. (777)	16	1
3**	49	pos (593)	16	0
4	12	pos. (1317)	19	0
4**	12	pos (1038)	19	3
5	1	pos. (885)	14	1
5**	1	pos. (1410)	14	2

* Donor time = interval between death and start of culture.
** Second cornea of a pair.

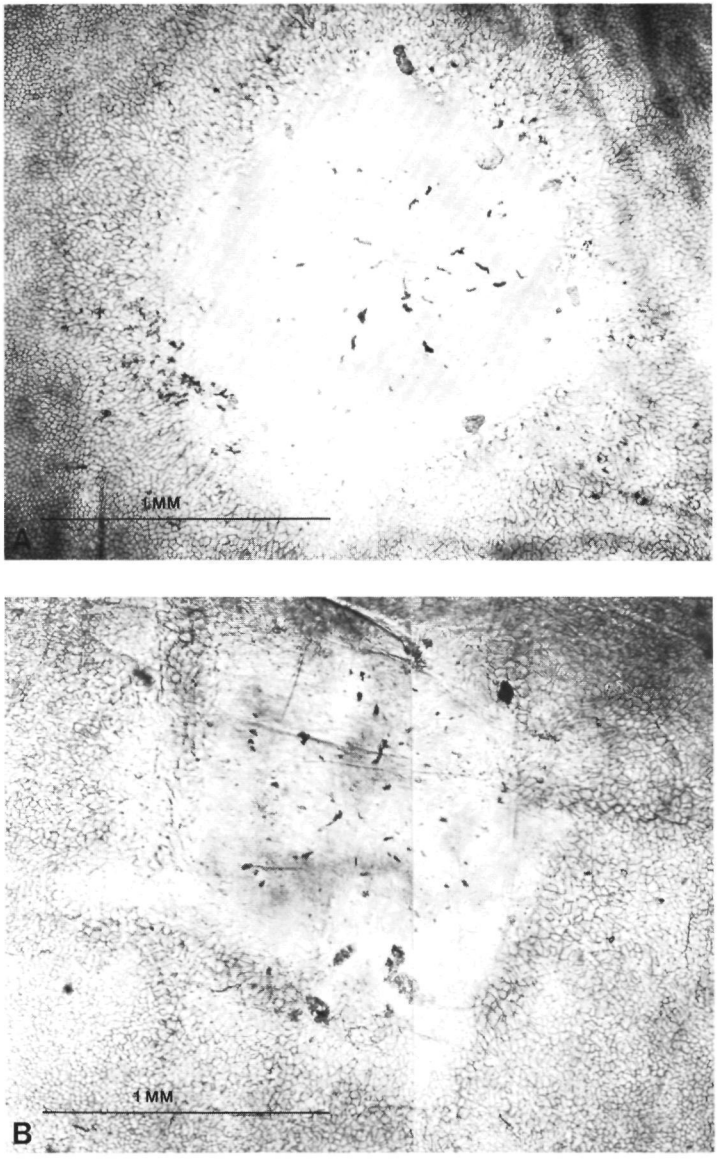


FIG. 40 *Silver staining of human corneas. Autoradiography and nuclear staining are still to be done. The regular endothelial cell pattern at the periphery changes via a zone of transition to the irregular pattern of the cells in the central wound area.*
A. Corneal wound after 1 day in vitro. Donor aged 71. Number of autoradiographically positive cells: 0. Counted after autoradiography was done.
B. Corneal wound after 2 days in vitro. Donor aged 65. Number of autoradiographically positive cells: 220. Counted after autoradiography was done.

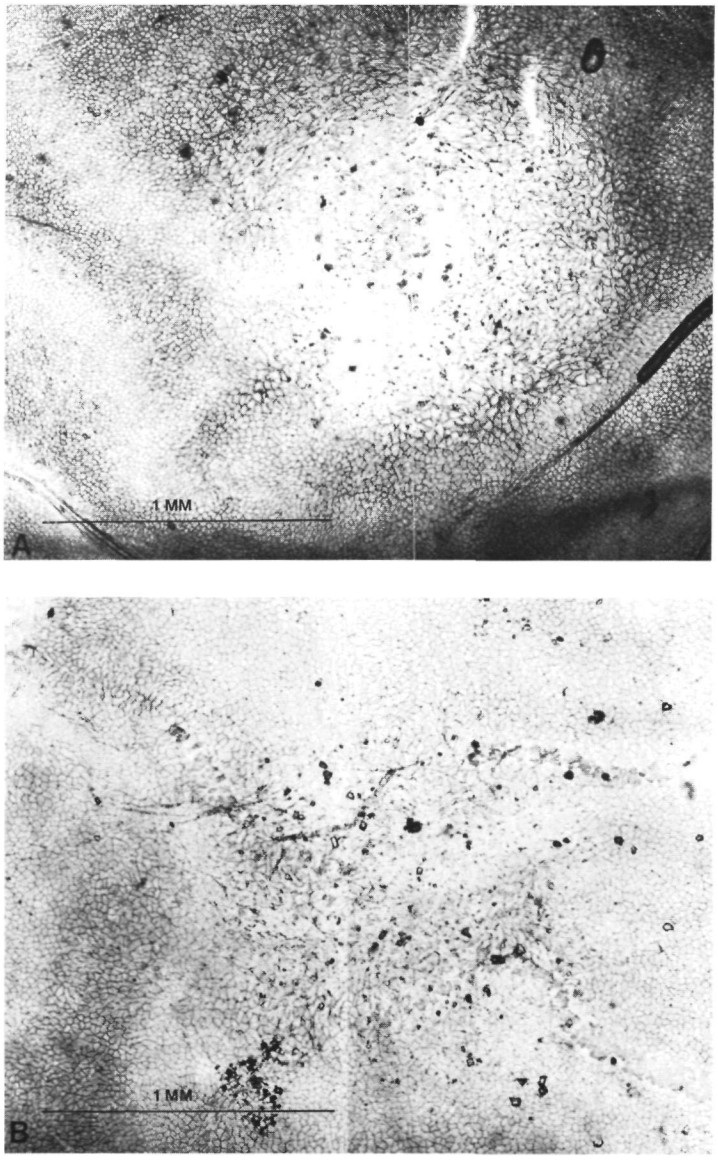


FIG. 41 *Silver staining of human corneas. Autoradiography and nuclear staining are still to be done. The regular endothelial cell pattern at the periphery changes via a zone of transition to the irregular pattern of the cells in the central wound area.*
A. Corneal wound after 3 days in vitro. Donor aged 52. Number of autoradiographically positive cells: 5. Counted after autoradiography was done.
B. Corneal wound after 4 days in vitro. Donor aged 60. Number of autoradiographically positive cells: 0. Counted after autoradiography was done.

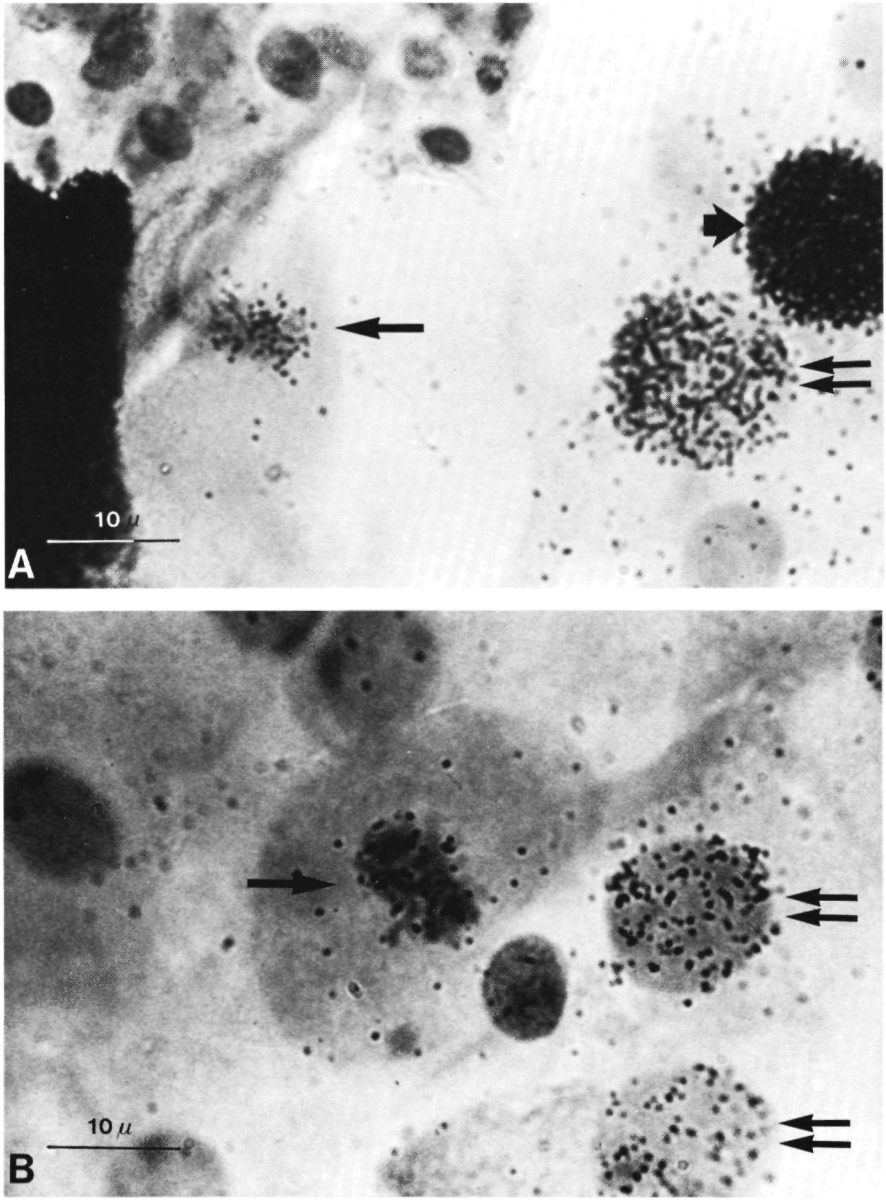


FIG. 42 *Autoradiography and nuclear staining of human cornea after 3 days culture. A and B show the same specimen. Two tritium thymidine positive mitotic figures (arrow). The cell membrane is clearly distinguishable because the nuclear staining also slightly stains the cytoplasm. DNA-synthesizing cells are also visible in the specimen (double arrow). Sometimes the density of the autoradiograph is too intensive for proper evaluation of the underlying nuclei with nuclear staining (heavy arrow). Donor aged 12.*

4.2.6 Qualitative difference in wound healing between a mechanical and a cryo-induced corneal endothelial lesion

The purpose of this experiment was to establish whether a qualitative difference in wound healing in paired corneas existed between a mechanical and a transcorneal cryo-lesion of the endothelium. The diameter of the cryo-probe used to freeze the cornea twice transcorneally for 20 seconds, was 2 mm. The experiment was done on paired corneas. Tritium thymidine was added to the culture medium (5 μCi/ml) one hour before completion of the culture (pulsed exposure).

Silver staining and nuclear staining revealed that the wound still had not closed after 24 hours. After 48 hours' culture the wound was closed in three of the four corneas. In all corneas cultured longer the wound was likewise found to have closed. Silver staining showed an irregular cell pattern at the centre of the wound area; next came a zone of transition, and at the periphery the cell pattern was normal.

Two pairs of corneas were not submitted to autoradiography (table 4.2.6).

TABLE 4.2.6 *Autoradiographic results in 20 human corneas cultured for 1, 2, 3 or 4 days. Figures in brackets indicate numbers of positive nuclei in the wound area. In each pair of corneas, one had a standard mechanical wound (smw) and the other a transcorneal cryo-lesion (tcl).*

Pair of corneas number		Age in years	Duration of culture 1 day	2 days	3 days	4 days	Donor time* in hours	Mitosis	Wound closed
1	smw	45	neg.				20	n.a.	no
1**	tcl	45	neg.				20	n.a.	no
2	smw	50	neg.				14	n.a.	no
2**	tcl	50	neg.				14	n.a.	no
3	smw	26		n.d.			20	pos.	yes
3**	tcl	26		n.d.			20	neg.	yes
4	smw	45		pos. (150)			19	n.a.	no
4**	tcl	45		pos. (120)			19	n.a.	yes
5	smw	42			n.d.		16	pos.	yes
5**	tcl	42			n.d.		16	neg.	yes
6	smw	25			pos. (95)		12	n.a.	yes
6**	tcl	25			neg.		12	n.a.	yes
7	smw	2			pos. (30)		18	n.a.	yes
7**	tcl	2			pos. (45)		18	n.a.	yes
8	smw	15			pos. (186)		12	pos.	yes
8**	*tcl*	15			pos. (13)		12	n.a.	yes
9	smw	67				pos. (31)	18	n.a.	yes
9**	tcl	67				neg.	18	n.a.	yes
10	smw	45				neg.	14	n.a.	yes
10**	tcl	45				neg.	14	n.a.	yes

* Donor time = interval between death and start of culture.
** Second cornea of a pair.
n.a. = not assessible.
n.d. = not done.

Mitotic figures were found in those two specimens with a mechanical endothelial lesion, but not in the fellow two with a transcorneal cryo-lesion. All other pairs of corneas were submitted to silver staining, combined with autoradiography and nuclear staining The autoradiograph was negative in all cases after 1 day, and positive after 2 days. Of the six corneas cultured for 3 days, five were autoradiographically positive; of the 4-day specimens, only one out of four corneas was positive (table 4.2.6).
In nearly all specimens submitted to autoradiography, the background density was too intensive to permit evaluation of the nuclear staining The exception was one specimen, in which eight mitotic figures were found

4.2.7 Influence of exogenous factors

The purpose of this experiment was to establish whether the observed proliferation of endothelial cells could have been induced non-specifically by the foetal calf serum used. This calf serum contains many unknown factors. which might stimulate proliferation.

TABLE 4 2 7 *Autoradiographic results in 16 human corneas cultured for 1, 2, 3 or 4 days Figures in brackets indicate numbers of positive nuclei in the wound area In each pair of corneas, one was cultured with and the other without foetal calf serum Standard wound*

Pair of corneas number		Age in years	Duration of culture 1 day	2 days	3 days	4 days	Donor time* in hours	Mitosis	Wound closed
1	fcs+	70	n d				15	neg	no
1**	fcs—	70	n d				15	neg	no
2	fcs+	35		pos (212)			16	neg	yes
2**	fcs—	35		pos (292)			16	neg	no
3	fcs+	34		n d			15	pos	yes
3**	fcs—	34		n d			15	neg	no
4	fcs+	16			pos (23)		16	pos	yes
4**	fcs—	16			pos (90)		16	pos	yes
5	fcs+	21			n d		15	pos	yes
5**	fcs—	21			n d		15	pos	no
6	fcs+	58			n d		13	pos	yes
6**	fcs—	58	culture infected						
7	fcs+	69				pos (30)	19	neg	yes
7**	fcs—	69				pos (6)	19	pos	yes
8	fcs+	31				n d	9	pos	yes
8**	fcs—	31				n d	9	pos	no

* Donor time = interval between death and start of culture
** Second cornea of a pair
fcs+ = medium with foetal calf serum
fcs— = medium without foetal calf serum
n d = not done

One cornea of a donor pair was cultured in a medium containing foetal calf serum, while the fellow cornea was cultured without this serum. The duration of culture per pair of corneas was the same (table 4.2.7). Eight donor pairs were thus treated. In both groups the endothelial wound had not closed after one day, but migration had started. The endothelial wounds in the corneas cultured with foetal calf serum had closed after 2, 3 and 4 days. In the corneas cultured without foetal calf serum endothelial wound closure was delayed, but did occur (table 4.2.7).
Three pairs of corneas were submitted to autoradiography, which was positive in all six specimens (table 4.2.7). Nuclear staining disclosed mitotic figures in both groups (figs. 43, 44, 45, 46).

4.2.8 Scanning electron microscopy of endothelial wound healing

The purpose of this experiment was to establish whether this way of studying wound healing would supply additional information with the different visualization of wound healing.

Hour 0 after trauma:
The specimen showed a circular endothelial wound with a sharply defined margin. The endothelial cells immediately adjacent to the wound margin showed some damage, but the more peripheral cells had a normal, regular cell pattern. The interdigitation of the apical folds at the apical cell boundary was clearly visible (fig. 47).

Day 1 after trauma:
The wound area had largely closed. The cells had lost their normal shape and were evidently irregular. This pattern gradually changed to the normal pattern at the periphery. The processes of the endothelial cells which had migrated into the wound were seen to cross each other.

Day 2 after trauma:
Crossing cell processes were observed in the now virtually closed wound area. The cells at the periphery of the cornea presented a normal appearance. Cell notchings indicating a cytokinetic event were not observed (fig. 48).

Day 3 after trauma:
The features of the wound area had no changed essentially since day 2. Crossing cell processes were observed. In other parts of the wound area, cell boundaries were seen where the apical folds were already interdigitating again. There was no evidence of cytokinesis, and the periphery showed a normal cell pattern.

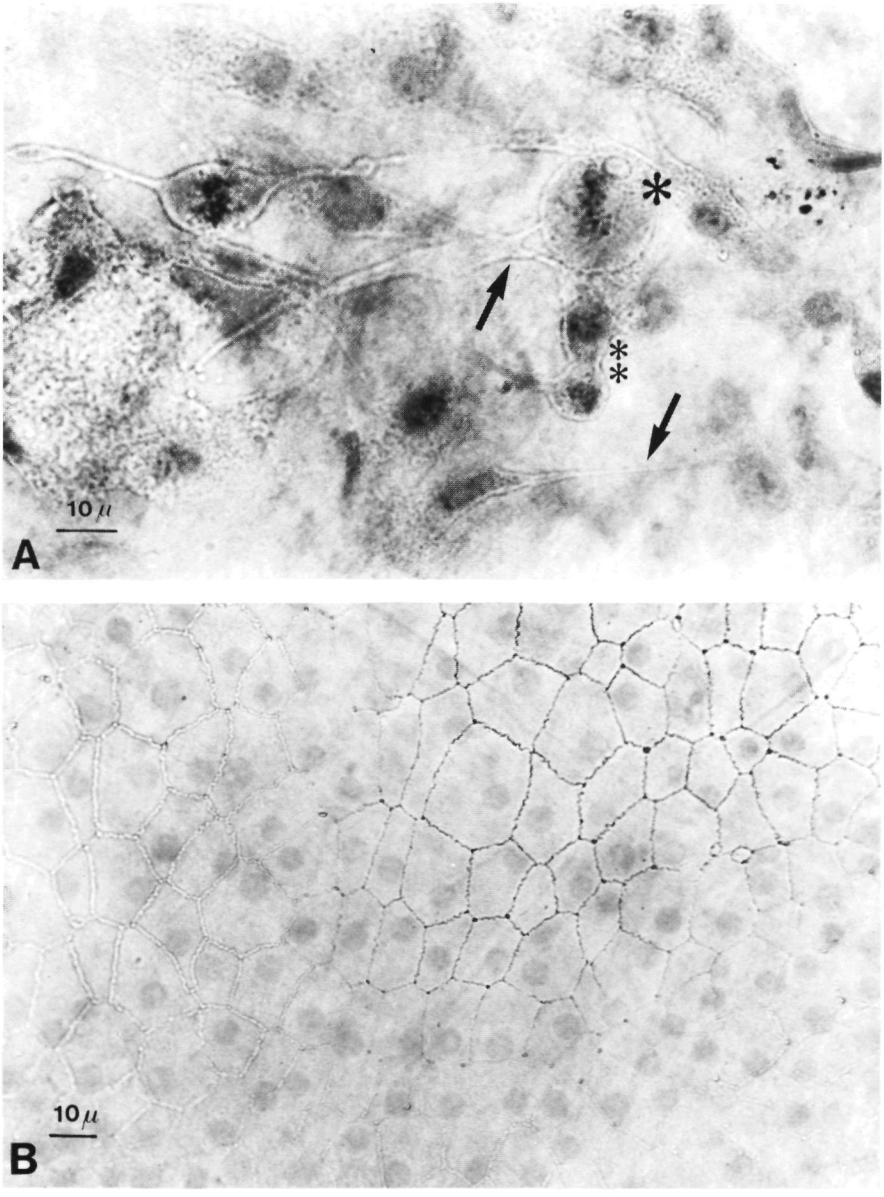


FIG. 43 *Silver staining and nuclear staining of human cornea after 3 days' culture. Figs. 43 and 44 show the same specimen.*
A. Central wound area in which the cell processes (arrow) which link the cells are visible. A metaphasic mitotic figure (asterix) and telophase (double asterix) are likewise visible.
B. Periphery of the same cornea. Each endothelial cell has one nucleus.
The culture medium contains no foetal calf serum. Donor aged 21.

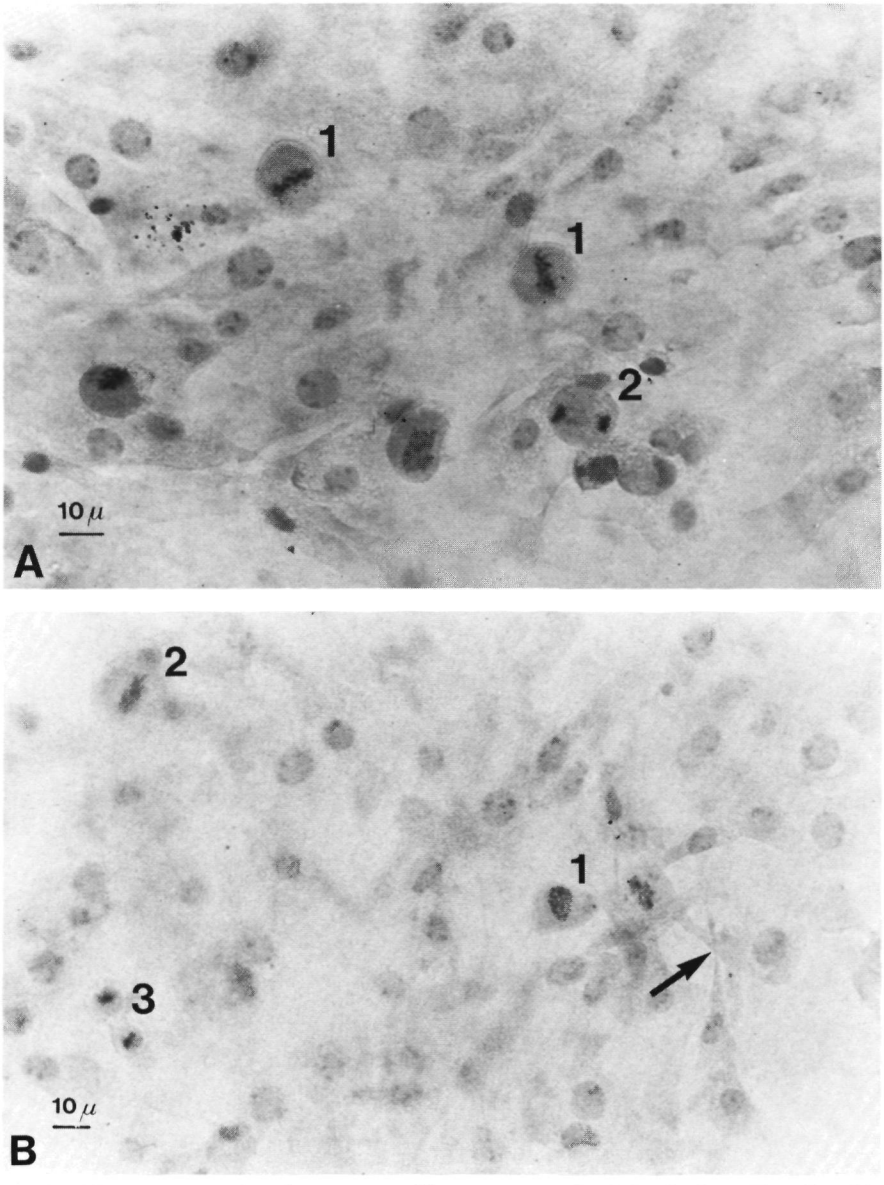


FIG. 44 *Silver staining and nuclear staining of human cornea after 3 days' culture. Figs. 43 and 44 show the same specimen.*
A. Central wound area in which various mitotic figures are visible. Metaphasic mitotic figures (1) and an anaphase (2) are visible.
B. Different part of the same wound area, in which long cell processes (arrow) are visible which cross each other. Also visible are a prophase (1), a metaphase (2) and a telophase (3).
The culture medium contains no foetal calf serum. Donor aged 21.

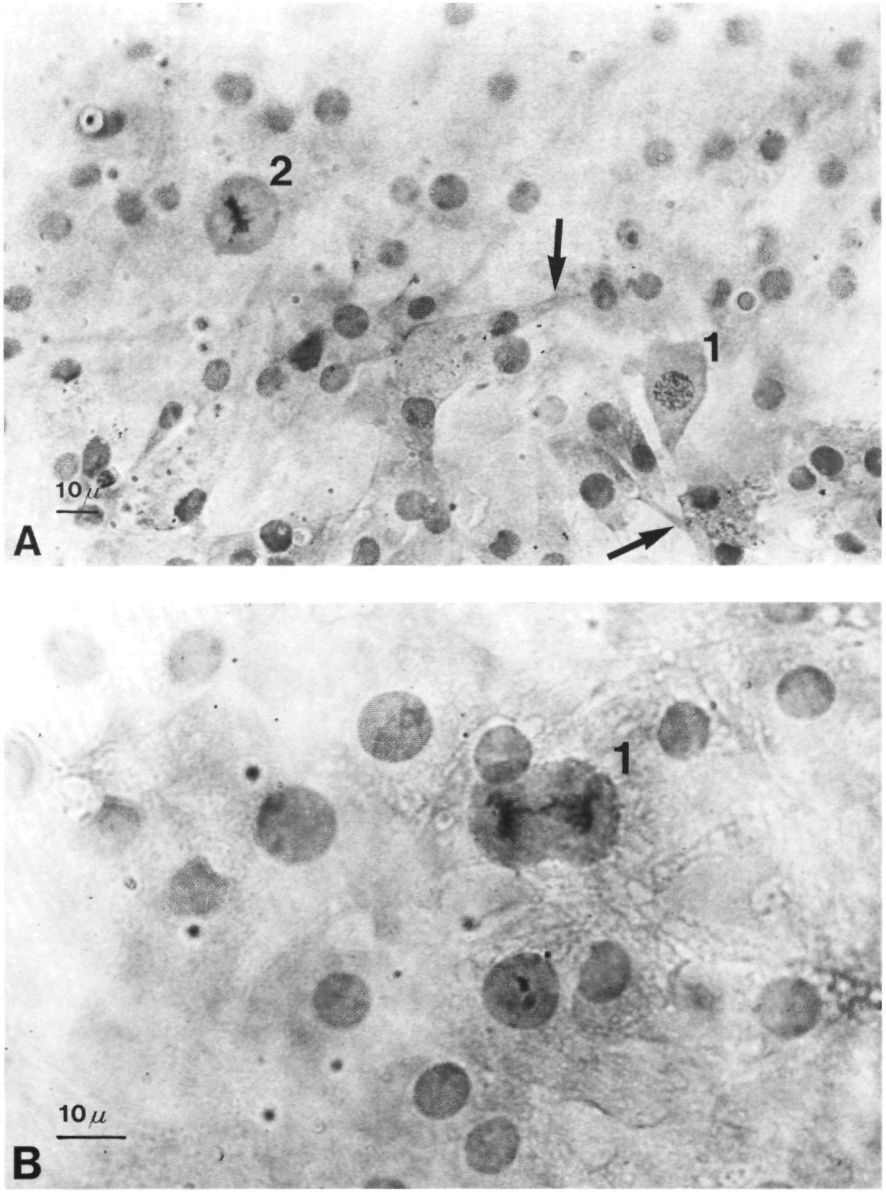


FIG. 45 *Nuclear staining of human cornea after 3 days' culture. Figs. 45 and 46 show the same specimen.*
A. Cells linked by cell processes (arrow) and mitotic figure, prophase (1) and metaphase (2).
B. Mitotic figure, anaphase (1).
The culture medium contains foetal calf serum. Donor aged 58.

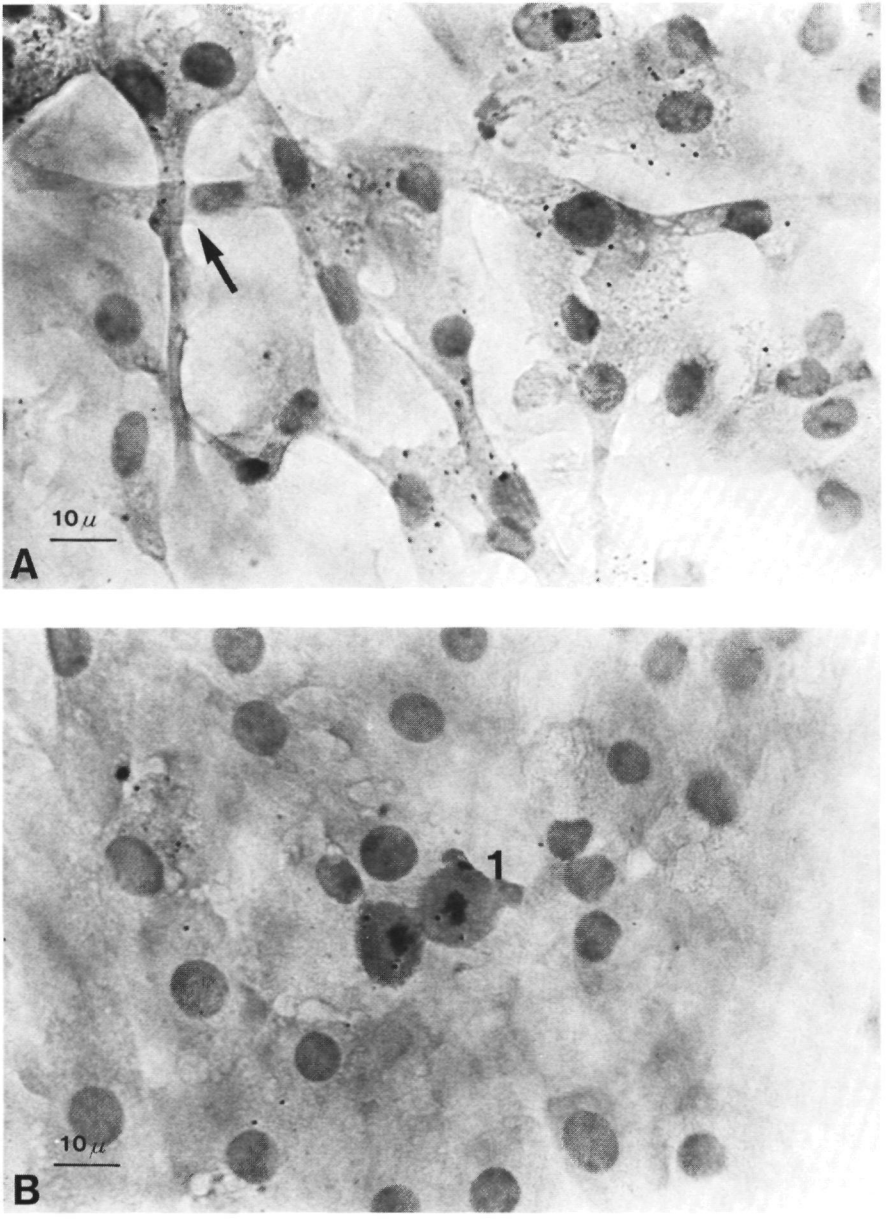


FIG. 46 *Nuclear staining of human cornea after 3 days' culture. Figs. 45 and 46 show the same specimen.*
A. Cell junctions in the central wound area; some cell processes cross each other (arrow).
B. Mitotic figure, telophase (1).
The culture medium contains foetal calf serum. Donor aged 58.

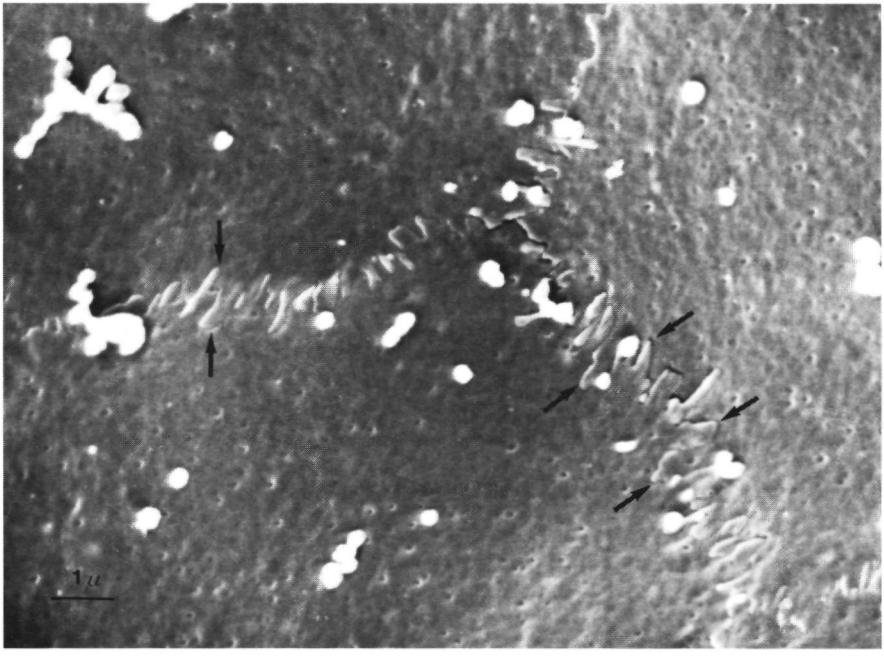


FIG. 47 *SEM of human cornea 0 hour after trauma. Magnification of an area localized beside the wound. Unmistakable interdigitation of the apical folds (arrow). Donor aged 29.*

Day 4 after trauma:
The cells in the wound area showed fewer cross-links than on previous days. A normal apical cell boundary was seen at some sites (fig. 49). The cells in the wound area showed a more uniform morphology, while those at the periphery presented a normal, regular appearance. No evidence of cytokinesis was found.

4.2.9 Cross-sections

In this experiment, corneal cross-sections were studied in order to establish the normal anatomy and histology of the endothelial wound. Silver-stained corneas (with and without wound) were also examined, in order to gain an impression of the localization of the silver deposit.

4.2.9.1 Normal endothelium

The following findings were obtained in normal corneal endothelium, without lesion and not stained with silver.

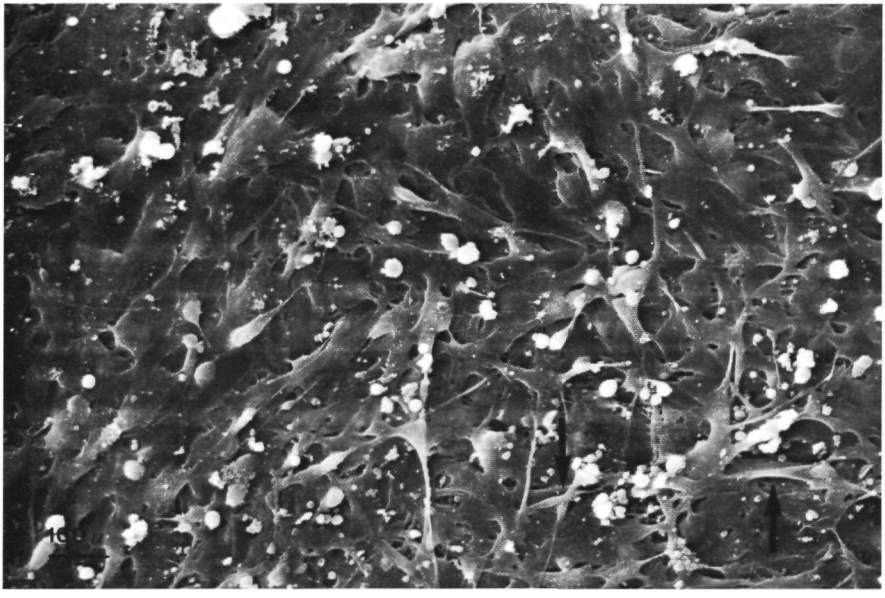


FIG. 48 *SEM of human cornea after 2 days' culture. The wound is virtually closed. The wound area is populated by cells with long processes that cross each other (arrow). Donor aged 30.*

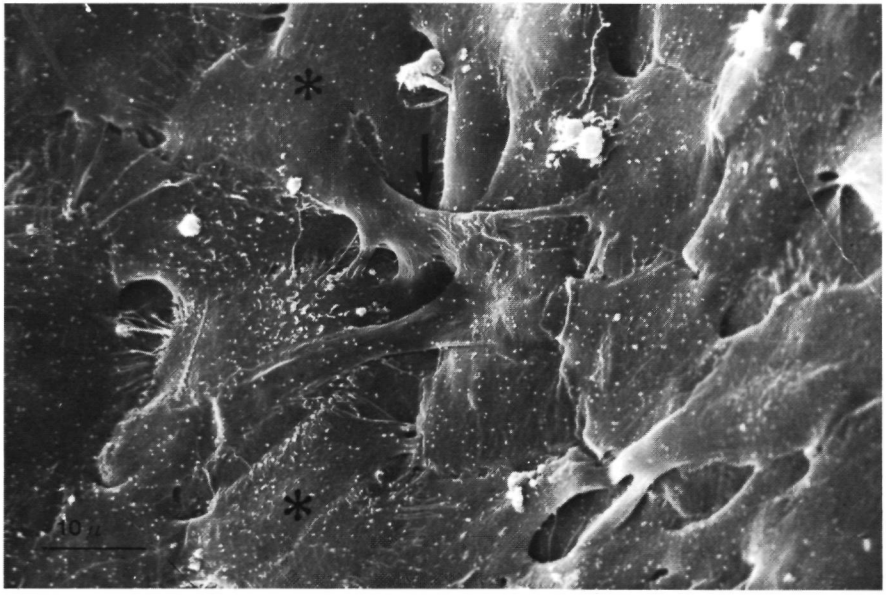


FIG. 49 *SEM of human cornea after 4 days' culture. The wound area is closed by cells which at some sites already show a normal apical cell demarcation (asterix). At other sites cell processes are still seen to cross each other (arrow). Donor aged 30. Compare with fig. 46 A and fig. 57.*

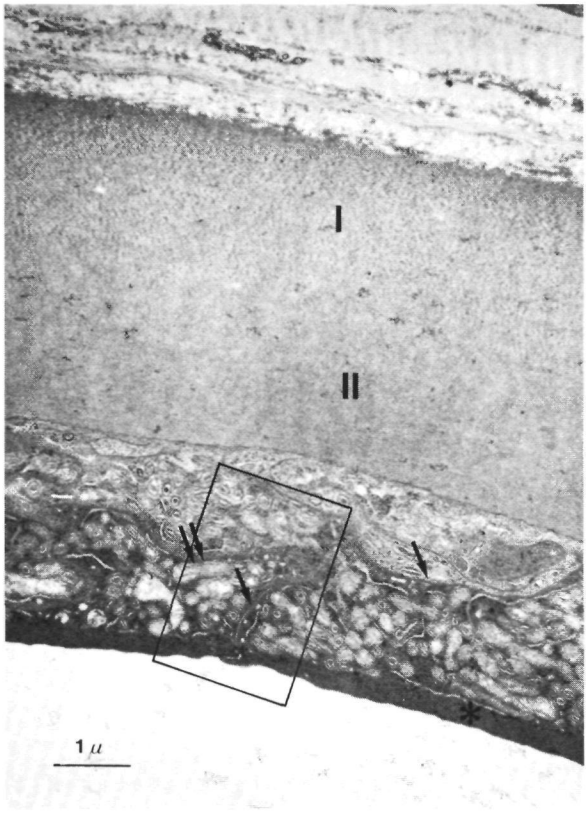


FIG. 50 *TEM of normal human cornea. Descemet's membrane and endothelium. The densification beneath the apical cell membrane is the terminal web (asterix). The interdigitation of the lateral cell membrane (arrow) and the numerous mitochondria (double arrow) are clearly visible. The two layers of Descemet's membrane - the older (I) and the younger (II) - are distinguishable. Donor aged 20.*

Light microscopy:
Descemet's membrane had about the same thickness as the endothelium. The endothelial cell nuclei were oval-shaped, sometimes flattened on one side, and were localized at the centre of the cell.

Transmission electron microscopy:
Two parts of Descemet's membrane were distinguishable. The anterior (older) part presented a vertical band-like appearance; the posterior (younger) part had more amorphous granular features (fig. 50).
The endothelium showed an apically localized dense, homogeneous intracel-

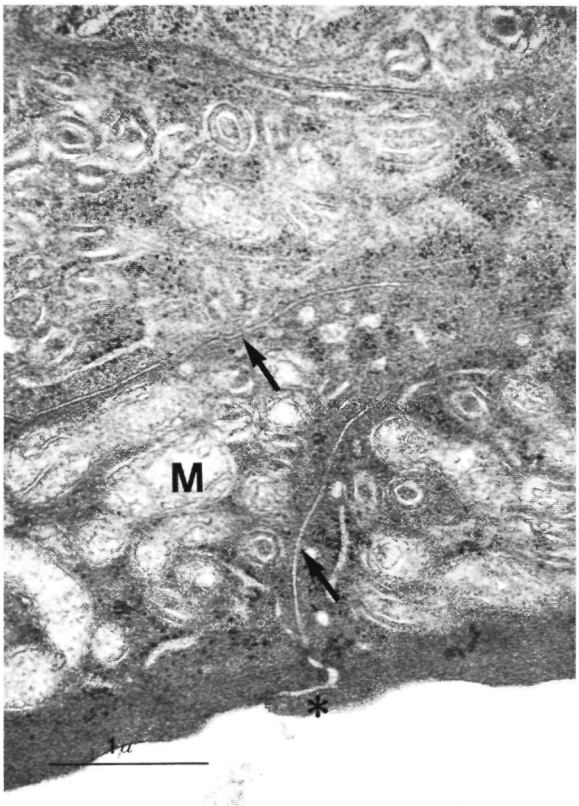


FIG. 51 *TEM of normal human cornea. Magnification of detail of fig. 50. Apical fold (asterix). The lateral cell membrane (arrow) with its tortuous course is clearly visible. The cytoplasm contains many mitochondria (M). Donor aged 20.*

lular structure: the terminal web. At the cell boundary this structure - together with the adjacent cell - formed the terminal bar (belt desmosome and tight junction). The mitochondria and the Golgi apparatus were visible in the cytoplasm. The apical fold was quite distinctly visible at the site of the terminal bar (see 2.2.7). A very tortuous course of the intercellular space and interdigitation with adjacent cells were the rule rather than the exception (figs. 50 en 51, compare fig. 2).

4.2.9.2 Normal endothelium at silver staining

Light microscopy:
Descemet's membrane showed no changes. In the silver-stained specimen the

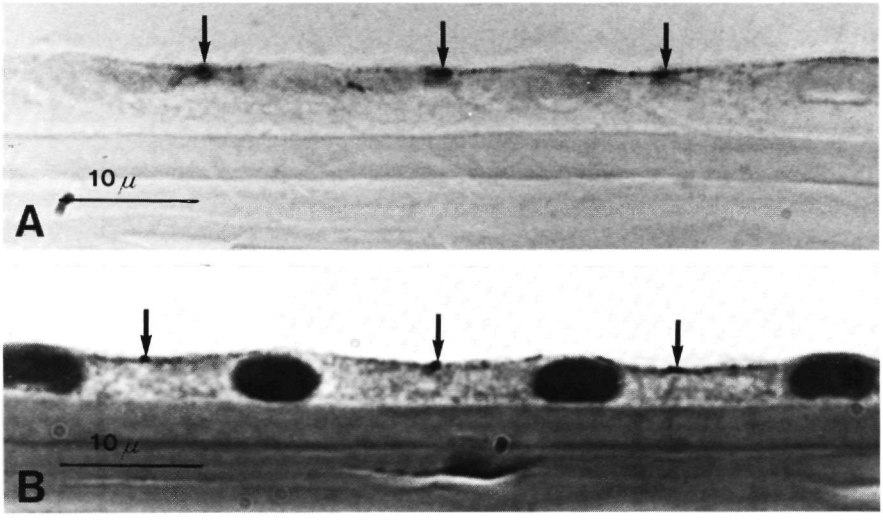


FIG. 52 *Normal human cornea, stained with silver (A) and after-stained with haematoxylin and eosin (B). The thin layer of silver on the apical cell membrane is more clearly visible in A. An intensive accumulation of silver particles is visible between the cell nuclei (arrow). Donor aged 20.*

endothelial cells were found to be covered with a thin layer of silver particles, showing some increased density at a few sites. The silver was localized on the cell surface (fig. 52A and 52B). Tight junctions prevented invasion of the intercellular space. A dark dot indicated the accumulation of silver particles beneath the apical fold which transmission electron microscopy (fig. 54) revealed. This accumulation was often localized between cell nuclei, and at some sites partly invaded the intercellular space. A similar silver localization was found in the specimens after-stained with haematoxylin and eosin, but in these specimens the normal cell structures were more clearly visible (fig. 52B). In the specimen not after-stained, however, the silver layer was more clearly visible (fig. 52A).

Transmission electron microscopy:
Much of the cytoplasmic structure had been lost in these sections, possibly as a result of the low pH of the silver nitrate solution (pH=5.6) in which the cornea was washed for 10 seconds as part of the silver staining procedure. Minute silver particles were visible on the apical cell surface (figs. 53 and 54). Translucencies and an occasional silver particle were visible at the site of the apical folds. The larger silver particles deposited were lost as a result of the processing (probably while the sections were cut), causing the translucency.

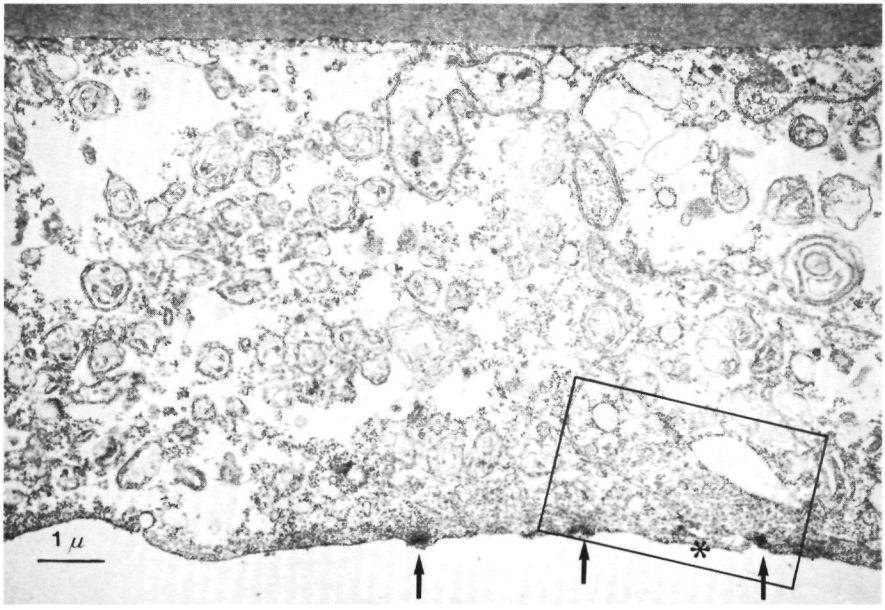


FIG. 53 *TEM of normal human corneal endothelium, stained with silver. The fine deposit of silver particles (arrow) is visible on the apical cell membrane. A defect in the section is visible beneath the apical fold (asterix). The silver accumulation was probably lost in the course of processing. Donor aged 32.*

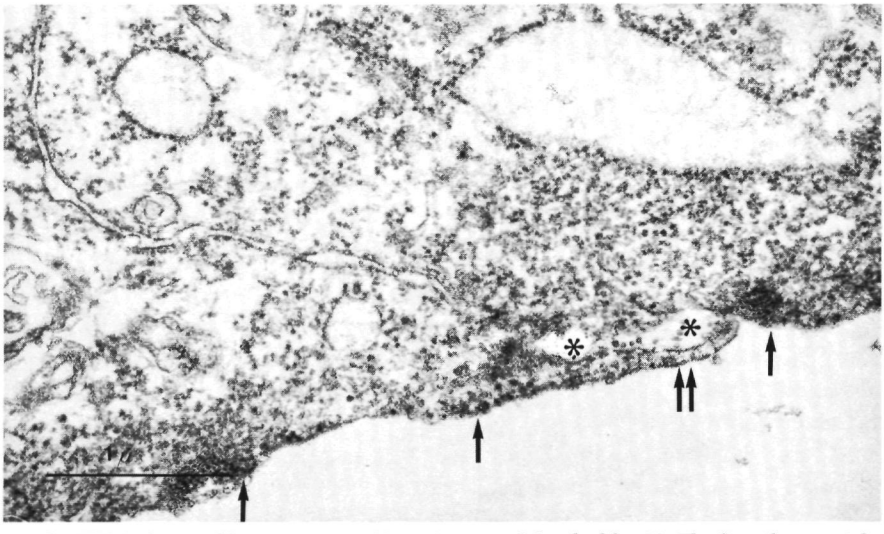


FIG. 54 *TEM of normal human cornea. Magnification of detail of fig. 53. The fine silver particles (arrow) are visible on the apical cell membrane. Beneath the apical fold (double arrow), a defect in the section is visible (asterix). The silver accumulation was probably lost in the course of processing. Donor aged 32.*

FIG. 55 *Autoradiograph of a cross-section of a human cornea after 3 days' culture. The densification of the film emulsion by the incorporated tritium thymidine is localized above the nucleus of the endothelial cell. The endothelial cells are slightly flattened. Descemet's membrane (asterix) is quite intact. Donor aged 41.*

4.2.9.3 Wound healing at silver staining

Corneas with a standard wound were cultured for 1 day and 2 days, stained with silver and then studied.

Transmission electron microscopy:
The apical cell surface showed numerous small evaginations and was covered with silver particles. These particles had also invaded the intercellular space, especially in the central part of the wound area where the intercellular spaces were widest and had not yet been closed by tight junctions. At some sites the silver nitrate even proved to have invaded Descemet's membrane. The cytoplasm showed severe damage, possibly as a result of the silver staining.

4.2.9.4 Wound healing at silver staining and autoradiography

Corneas with a standard wound were cultured for 3 days, with pulsed exposure to tritium thymidine, stained with silver and then studied.

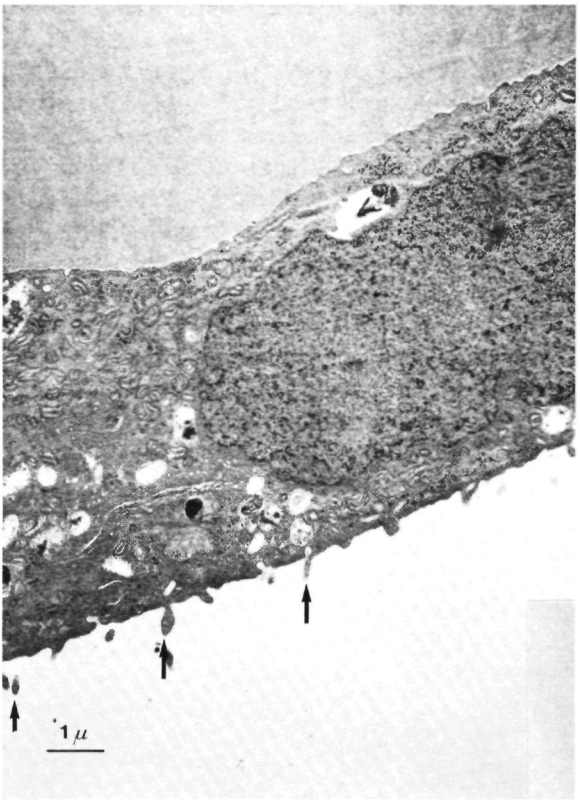


FIG. 56 *TEM of human cornea with standard endothelial wound after 3 days' culture. Microvilli on the apical cell membrane of the endothelium (arrow). Donor aged 41.*

Light microscopy:
The silver particles were localized mainly on the apical cell membrane. At some sites the silver nitrate had advanced between the cells to invade Descemet's membrane, particularly in the central part of the wound area. Autoradiography revealed that it was evidently the nucleus of the endothelial cell that synthesized DNA (fig. 55). Descemet's membrane was entirely intact.

Transmission electron microscopy:
The cytoplasmic structure was severely damaged. Small evaginations of the cell membrane were visible (fig. 56), as was the crossing of endothelial cell processes in the wound area (fig. 57). Wider intercellular spaces were found in the central part of the wound area.

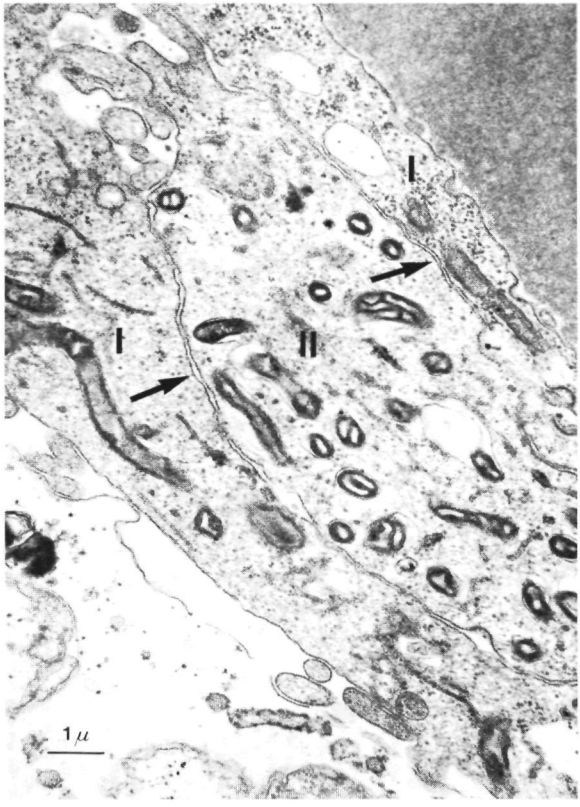


FIG. 57 *TEM of human cornea with standard wound after 3 days' culture. The cell processes of two endothelial cells (I and II) are seen to cross in the central wound area. The lateral cell membrane is clearly visible (arrow). Donor aged 41.*

4.3 IN-VIVO HUMAN CORNEAL ENDOTHELIAL WOUND HEALING

The final part of the study concerned in-vivo human corneal endothelial wound healing. Two patients who were to undergo enucleation volunteered for this experiment, and gave their informed consent in writing. In both, a transcorneal cryo-lesion of the endothelium was induced. Endothelial wound healing was then studied by the technique described.

I am especially indebted to E.W.D. Norton, M.D., who helped to recruit these two patients for the experiment, and to H.W. Flynn Jr, M.D. and S.L. Jaben, M.D. of the Bascom Palmer Eye Institute, Miami, Florida, USA, who permitted their patients to participate.

Patient 1

Patient 1 was a 67-year-old white male, who was to undergo enucleation of his right eye in view of the presence of a malignant melanoma of the choroid. The cornea was entirely normal at slit-lamp examination. There was no sign of cornea guttata.

A transcorneal cryo-lesion of the central corneal endothelium was induced 72 hours before the planned enucleation. The inflicting of the wound was done under topical anaesthesia. (febr. 27, 1981). The timing was based on the fact that the experiments with the primates and the cultured human corneas had shown that 72 hours was the interval after which DNA synthesis and mitotic figures were most likely to be found.

After cryocoagulation, only a pressure bandage was applied to the eye. Six hours after the intervention the patient complained of fairly intensive pain in the eye. In order to minimize the possible effect of medication on wound healing, a single application of a topical anaesthetic was given. The pain disappeared immediately. Twelve hours later (i.e. 18 hours after the intervention) the patient noticed no more than a slight inconvenience.

On the day of the enucleation (march 2, 1981) the eye showed central stromal oedema with fine folds in Descemet's membrane. The aqueous humour contained a moderate amount of cells (+) and flare (+). Specular microscopy was impossible due to the stromal oedema.

After enucleation of the eye and dissection of the cornea, the cornea was incubated for one hour in vitro at 37°C in the medium described (see 3.2.1), which contained tritium thymidine (5 μCi/ml). After this hour silver staining was done, followed by fixation in 5% glutaric aldehyde at 4°C, autoradiography and nuclear staining. The maximum interval between enucleation and fixation was 2 hours.

Silver staining revealed a normal, regular endothelial pattern at the corneal periphery. The cell pattern gradually changed in central direction. The most peripheral cells in this zone of transition began to arrange themselves radially in the direction of the wound. Further towards the centre there was a gradual change to more irregular cells. The wound proper contained endothelial cells with an irregular appearance (fig. 58).

In order to minimize corneal curling, only the central 9 mm part of the cornea was used for autoradiography. The number of DNA-synthesizing cells in the wound area was 76 (fig. 59, compare with table 4.2.4 and survey C). Not a single positive cell was found at the periphery of the specimen, where the cell pattern was regular. Nuclear staining revealed a few mitotic figures in the central wound area (fig. 60). The nuclei of the cells in the wound area and the transitional zone were larger than those of the cells at the periphery.

Patient 2

Patient 2 was a 65-year-old black female, who was to undergo enucleation of

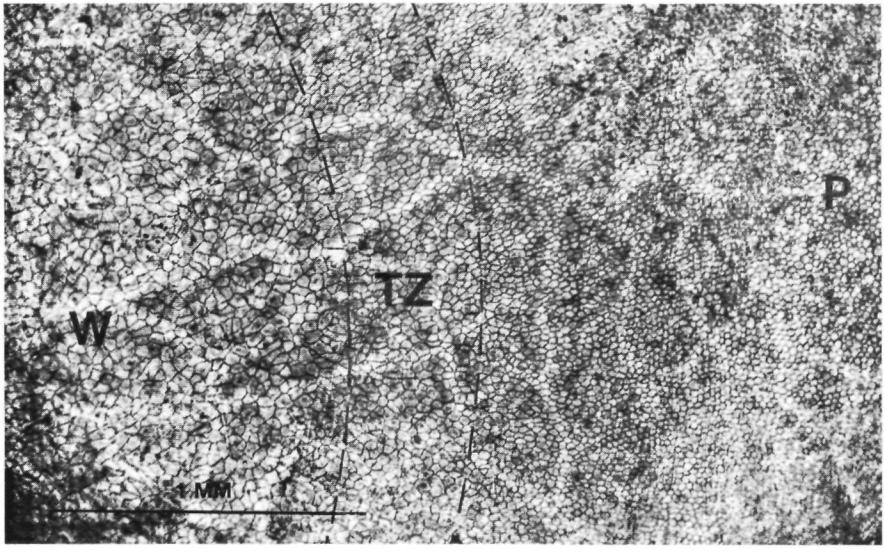


FIG. 58 *Silver staining of human cornea 3 days after cryo-induced lesion in vivo. The normal regular endothelial pattern at the periphery (P) changes via a transitional zone (TZ) to an irregular cell pattern in the wound area (W). Patient 1, aged 67. Autoradiography and nuclear staining are still to be done.*

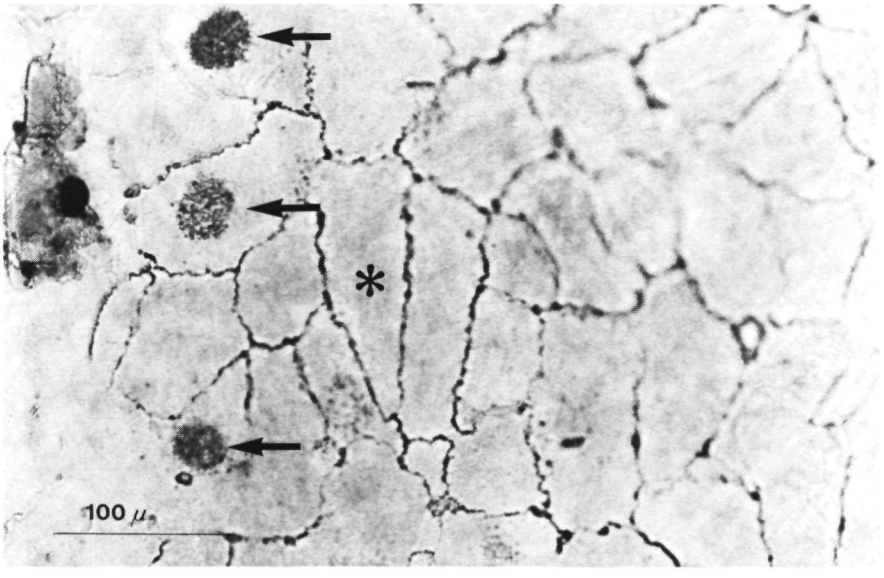


FIG. 59 *Silver staining, autoradiography and nuclear staining of a human corneal endothelial wound in vivo. Unmistakable irregular endothelial cell pattern in the central wound area (asterix). A few autoradiographically positive cells (arrow). Patient 1, aged 67, 3 days after transcorneal cryo-lesion of the endothelium in vivo.*

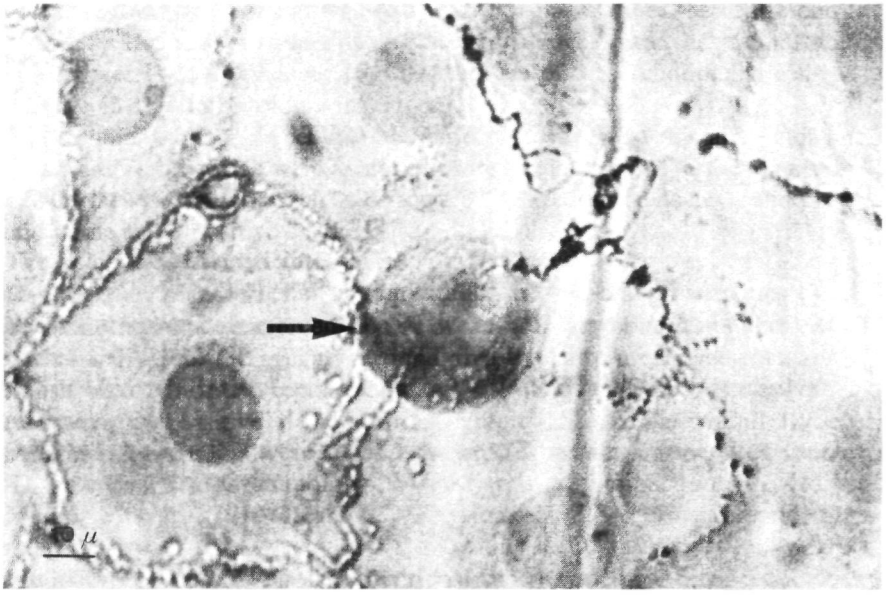


FIG. 60 *Silver staining, autoradiography and nuclear staining of a human corneal endothelial wound in vivo. A metaphasic mitotic figure (arrow) is found in an different part of the central wound area than that shown in fig. 59.*

her right eye in view of the presence of a malignant melanoma of the choroid. Slit-lamp examination revealed a few guttata spots at the centre of the cornea. No other changes were found in the anterior segment.

The transcorneal cryo-lesion of the endothelium (march 11, 1981) was induced 48 hours before the planned operation, in the way described for the first patient. Stromal oedema was observed in the wound area 18 hours after freezing: the deep stromal layers presented a whitish appearance (fig. 61), and showed a few keratic precipitates and folds. The anterior chamber contained a few cells and some flare.

There was also an erosion of corneal epithelium, which caused but little subjective inconvenience (as did the intervention itself). The epithelial erosion had healed 42 hours after freezing, but the stroma was still oedematous. At this time the aqueous humour contained numerous cells (++) and much flare (++). In view of cardiac arrhythmia during induction of general anaesthesia, the operation was postponed 72 hours.

On the day of the operation (march 16, 1981), i.e. 120 hours after induction of the cryo-lesion, the stromal oedema had largely disappeared (fig. 62). The deep stromal layers still showed greyish discoloration, which could only be observed with the aid of indirect illumination.

The procedure which followed enucleation of the eye was the same as that in the first patient, as was the histological technique used.

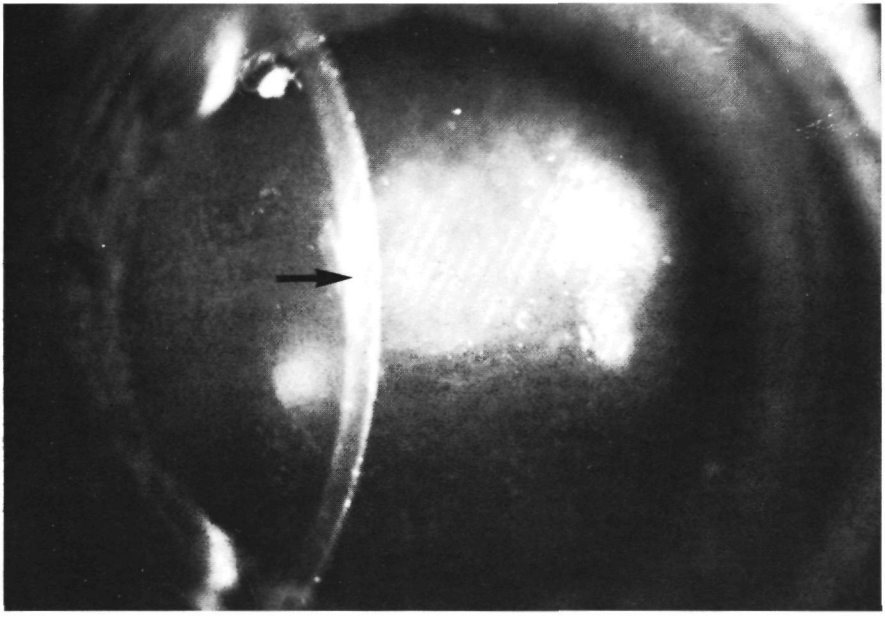


FIG. 61 *Slit-lamp photograph of the cornea 18 hours after transcorneal endothelial trauma. The stroma is swollen and whitish (arrow). Patient 2, aged 65. Wound healing in vivo.*

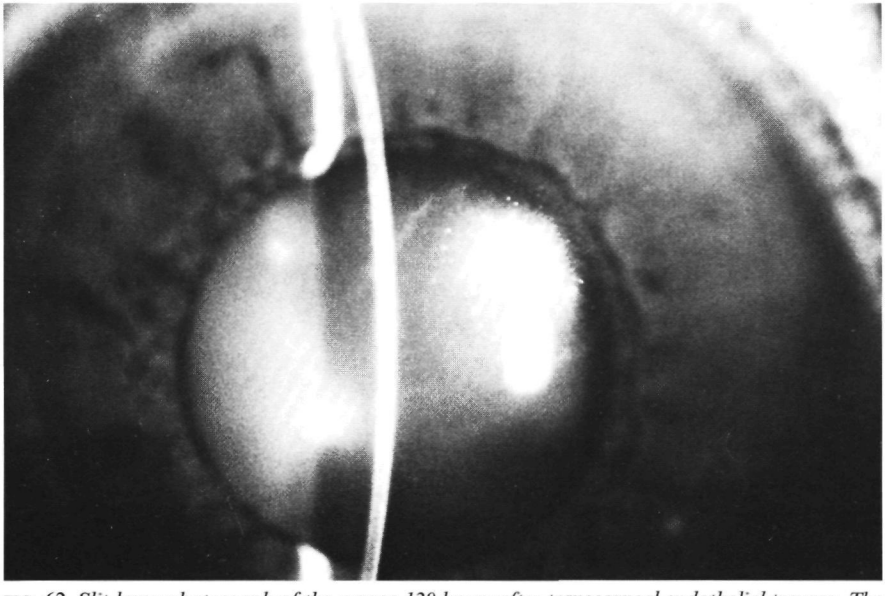


FIG. 62 *Slit-lamp photograph of the cornea 120 hours after transcorneal endothelial trauma. The cornea has virtually regained its normal thickness. Compare with fig. 61. Patient 2, aged 65. Wound healing in vivo.*

Silver staining showed that the endothelial wound had largely closed. The endothelial configuration was regular at the periphery. The central wound showed an irregular endothelial pattern comparable to that observed in the first patient. Centre and periphery were separated by a transitional zone. Again, only the central 9 mm part of the cornea was used for autoradiography. Specular microscopy was now possible, and revealed large irregular cells at the centre of the corneal wound area, whereas at the periphery the endothelial cells showed a regular pattern and were smaller (fig. 63).

Autoradiography revealed that 30 cells in the wound area had synthesized DNA during the hour of exposure of the wound to tritium thymidine in vitro (5 μCi/ml). In none of the endothelial cells at the periphery of the specimen DNA synthesis was observed. One mitotic figure was found. The nuclei of the cells in the wound area and transitional zone were larger than those of the cells at the periphery. Some nuclei of cells at the wound centre were pyknotic. They were found in a part of the wound which had not yet stained with silver.

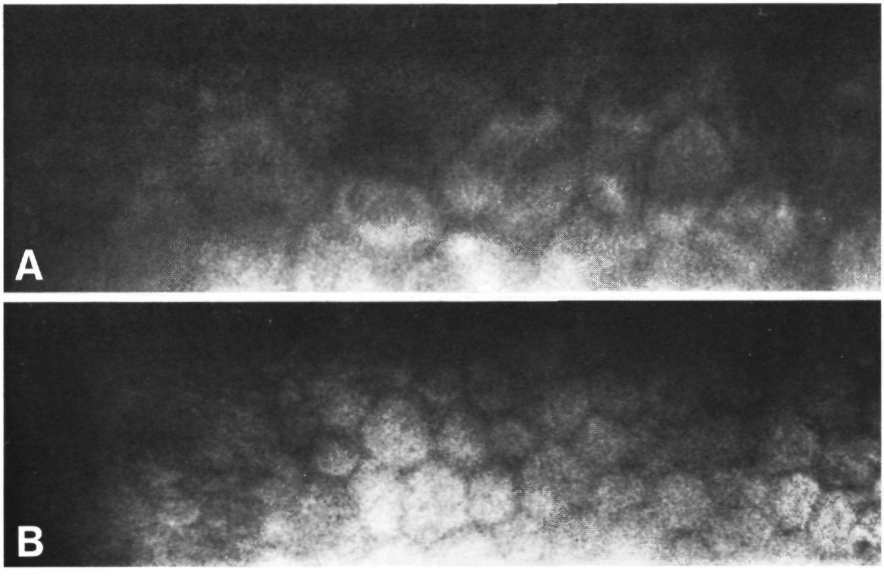


FIG. 63 *Specular microscopy of human corneal wound 120 hours after transcorneal cryo-lesion. A. Endothelial features in the central wound area. The cells are large and irregular. B. Endothelial features of an area outside the wound. The endothelial cells are smaller and more regular. Patient 2, aged 65. Wound healing in vivo.*

CONCLUSIONS AND DISCUSSION

Failure of corneal grafts can have a variety of causes, and the problem in diagnosis is differentiation between immunological and non-immunological factors. In actual practice, the decision is made on the basis of clinical experience.

The final result of grafting is partly determined by the wound healing process. Rapid, effective wound healing, and more specifically re-endothelialization, exerts a favourable influence on the prognosis of corneal grafts (Witmer 1980). The histopathology of failed penetrating human corneal grafts often reveals disturbances in deep-layer wound healing, frequently associated with retrocorneal membrane formation (Chi et al. 1962; Hales and Spencer 1963; Rycroft 1965; Brown and Kitano 1966; Kurz and D'Amico 1968; Winter 1969; Smith 1970; Kanai et al. 1972; Hinzpeter and Naumann 1977; Knöbel and V. Damarus 1979; Lahav and Cadet 1979). Clinically, retrocorneal membrane formation is often found in corneal grafts with less-than-optimal wound healing of the deep layers (Lemp 1976; Hinzpeter and Naumann 1977).

For specific research into the healing of human corneal grafts, animal models should be available which reflect the human situation as faithfully as possible. The current view is that corneal endothelium of primates, including man, is incapable of proliferation as part of wound healing (Kaufman et al. 1966; Stocker 1971; Capella 1972; Van Horn and Hyndiuk 1975; Doughman et al. 1976; Kaufman and Katz 1977; Gospodarowicz et al. 1979; Renard et al. 1981), whereas the corneal endothelium of young adult rabbits has this ability (Mills and Donn 1960).

Since the amount of endothelial cells which finally survive the operation correlates with the ultimate success of a graft, the extent and localization of peroperative endothelial cell loss was first studied. This was done with the aid of scanning electron microscopy and vital staining with trypan blue. This dye stains only damaged cell membranes (Stocker et al. 1966; Stocker 1971; Wickham and Binder 1976) and Descemet's membrane. The reliability of trypan blue in identifying cellular damage was demonstrated by Robbins et al. (1965) in a comparative study with a non-vital stain: nitro blue tetrazolium. It was found that both scanning electron microscopy and vital blue staining (compare figs. 9 and 10) showed that the damage with this operative technique was confined to the wound margin. These findings were consistent with those of a study in rabbits (Schimmelpfennig 1982).

In a limited number of animals, endothelial wound healing was studied in some detail with the aid of scanning electron microscopy and trypan blue

staining. It was found that the denuded part of Descemet's membrane, along the wound margin, rapidly re-endothelialized: in 3-5 days. This was confirmed by other investigators (Kaye et al. 1962; Polack et al. 1964; Faure et al. 1971; Olson et al. 1978).
The re-endothelialized part shows an irregular cell pattern with gradual re-arrangement to a more uniform pattern (Capella 1972; Hirsch et al. 1975; Van Horn and Hyndiuk 1975; Sherrard 1976; Van Horn et al. 1977; Rao et al. 1978, 1980; Yano and Tanishima 1980; Renard et al. 1981; Mishima 1982).
Re-endothelialization of the stromal part of the wound was much slower, in agreement with findings reported by Polack et al. (1964) and Ruusuvaara (1980). The absence of the physiological substrate must play a role in this respect (Gospodarowicz and Ill 1980). The difference in re-endothelialization of the stromal part of the wound in the same animal can be explained by a difference in wound adaptation (fig. 20). This was also observed by Landolt (1980) in a study of wound healing after cataract operations.

Good wound adaptation, particularly in the deeper layers, is of great importance for rapid re-endothelialization (Heller et al. 1971; Sanchez et al. 1974; Eve and Troutman 1976). A surgical technique purposefully focused on this is of vital importance. Although this technique involves more manipulation (with probably more endothelial loss), it is conceivable that the better adaptation of the deep layers is more important.
The results of the wound healing study prompted the question of the mechanisms underlying the endothelial healing process. Does this involve only migration, as generally assumed, or a proliferative aspect as well, as virtually all healing processes do?
To study this question, a histological technique was used which had not been previously described as a combination and which made it possible to study migration as well as proliferative features in the same specimen. Silver staining indicates the apical cell boundary, provided there are sufficient cell junctions between the cells (experiment 3.1.2.9). This makes it possible to study cell migration. The proliferative aspect was studied by demonstrating DNA synthesis (autoradiography) of incorporated tritium thymidine; in some cases this was combined with nuclear staining to demonstrate mitotic figures.
This technique provided more information than scanning electron microscopy (Renard et al. 1981; experiment 3.1.2.8) and scanning electron microscopy combined with autoradiography (Schutten et al. 1980). Moreover, scanning electron microscopy is more elaborate, expensive and time-consuming. Finally, the histological technique described in this study made it possible to study endothelial wound healing in a combined stromal and endothelial wound.
The experiments in non-human primates revealed that endothelial wound healing occurred in vivo and that migration as well as proliferation was involved (experiments 3.1.2 and 3.1.3). This finding confirmed reports by Gloor et al. (1980) and Svedbergh (1975). The endothelial healing process

showed a similar morphological aspect in both experiments, although the two groups differed in that the one in experiment 3.1.2 had a purely endothelial lesion while that in experiment 3.1.3. had a stromal as well as an endothelial lesion.
The course of wound healing can be described as follows. Immediately after infliction of the wound, migration of endothelial cells started, resulting in a radial arrangement of endothelial cells round the wound margin (figs. 24 and 27). DNA synthesis started after a latent period of about 24 hours, and was confined to the wound area proper. DNA synthesis diminished as soon as the wound was closed (experiment 3.1.2) or after 5-6 days, even if re-endothelialization had not yet been completed (experiment 3.1.3). The endothelial cells in the wound area showed an irregular pattern. In experiment 3.1.3, in which a stromal wound existed as well, DNA synthesis was observed in the stromal part of the wound on the 6th day. In view of their arrangement, these cells probably originated from the stroma.
Disturbed endothelial continuity, e.g. in a blood vessel after inplantation of a vascular prosthesis, causes the endothelial cells to regenerate rapidly over large distances of the inner surface, until proliferation and migration are inhibited and finally arrested as a result of contact inhibition (vide infra) (Hess et al. 1982).
The animal experiments described in this thesis revealed that disturbance of endothelial continuity was followed by rapid migration, supported by somewhat slowly starting proliferation. When the endothelial wound closed, proliferation diminished as a result of contact inhibition. The term 'contact inhibition' comes from cell cultivation work and implies the cessation of movement of animal cells as soon as they come into contact with each other. This contact, which occurs when separately growing islets of the monolayer start to become confluent, plays a role in the diminution of mitotic activity. This diminution of mitotic activity also depends on other factors (Schutz and Mora 1968; Yeh and Fisher 1969; Delbucco 1970; Martz and Steinberg 1974; Folkman and Moscona 1978; Zetterberg and Auer 1979). Stoker and Rubin (1967) proposed the term 'contact inhibition' for the arrest of cellular movements, and described the diminution of mitotic activity as 'density-dependent inhibition'. This suggestion, however, is not always consistently followed.
The pattern of corneal endothelial wound healing observed in experiments on adult monkeys differs from the generally accepted view. The findings in these experiments were more consistent with corneal endothelial wound healing as found in rabbits, young and old.
Recent research had demonstrated that, contrary to the current views on corneal endothelial healing, the human cornea in vitro is indeed capable of proliferation. Simonsen et al (1981) demonstrated in vitro DNA synthesis as a feature of corneal endothelial wound healing. Zagorski (1980) described

mitotic figures, and Squires and Weimar (1980) did so too. The latter authors stimulated wound healing with growth factors.
Autoradiography, although an indirect method of demonstrating proliferation, is used in actual practice as a method to study mitotic activity of tissues. In this technique, tritium thymidine is used to demonstrate that a cell has attained the S-phase of the cell cycle (see 2.4.1), and synthesizes DNA required for cell division. Demonstration of mitotic figures, however, provides direct evidence that the cell has attained the M-phase.
In higher animal species the S-phase of a cell cycle is in fact always followed by an M-phase (Ham and Cormack 1979; Karp 1979), the daughter cells coming to rest in the G_1-phase or again participating in a cell cycle. Gelfant (1963) described a G_2-phase arrest in mouse epidermis. He regarded this as part of the healing mechanism, to be activated if necessary.
The histological technique described - silver staining to visualize migration, autoradiography to demonstrate DNA synthesis, and sometimes nuclear staining to demonstrate mitotic figures - was used to study in-vitro human corneal endothelial wound healing.
The equipment described made it possible to standardize the endothelial wound so that the various results could be compared.
The experiments performed to study in-vitro human corneal endothelial wound healing after continuous (3.1.2.3) and pulsed (3.1.2.4) exposure to tritium thymidine, clearly demonstrated DNA synthesis and mitotic figures as features of the wound healing process. Experiment 3.1.2.5 revealed autoradiographically positive mitotic figures and thus clearly demonstrated that the direct and the indirect technique to show proliferation were in agreement. This means that cells which show mitosis during the culture period must have incorporated the radioactive tritium thymidine label; in other words: the S-phase of these cells was followed by an M-phase. In the control experiment (3.1.2.2) with continuous exposure to the label but without infliction of an endothelial wound, no DNA synthesis was observed in the central corneal endothelium.
Experiment 3.1.2.7 excluded the possibility of non-specific stimulation of proliferation by exogenous factors contained in the foetal calf serum which was added to the culture medium with one, but not with the other cornea of each pair. In this was, a possible individual difference in proliferative potency between donors was excluded as well. Preparatory to in-vivo study of corneal endothelial wound healing, experiment 3.1.2.6 was performed in order to establish whether a qualitative difference might be found between a transcorneal cryo-induced endothelial lesion and a standard mechanical endothelial wound. This was considered necessary because in the in-vivo study it would be impossible to induce a mechanical endothelial lesion without invading the eye. Transcorneal cryo-lesion of the endothelium would be the only feasibility in in-vivo studies.

The results of this experiment (4.2.6) revealed no qualitative differences in wound healing.
In view of the results of the in-vitro experiments, it was decided to perform the in-vivo examination at a time at which proliferative activity was most likely to be visible: 2-3 days after infliction of the endothelial wound. This meant that the endothelial wound had to be inflicted 2-3 days before the scheduled enucleation of the eye. Due to unforeseen circumstances (see 4.3), wound healing was in actual fact studied 3 and 5 days after infliction of the wound, respectively. The tritium thymidine labelling of cells possibly in the S-phase was done in vitro (see 3.1.3).
The results obtained show that human corneal endothelium in vivo is certainly capable of proliferation in response to a lesion, even at an advanced age (the two participants were 65 and 67 years old).
Close similarities in the pattern of corneal endothelial healing were observed in human in-vitro and in-vivo studies. The pattern showed the same sequence and the same morphological characteristics as non-human corneal endothelial wound healing in vivo (compare; fig. 24; non-human primate, fig. 34; human cornea in vitro and fig. 58; human cornea in vivo).
How can we explain the hitherto generally accepted view that the corneal endothelium of primates (including man) is incapable of proliferation as a feature of wound healing? The test animals used initially were young adult rabbits, in which it was subsequently established that spontaneous cell divisions and DNA synthesis occurred in the corneal endothelium. It was therefore not very surprising that proliferative activity was also observed in wound healing in these animals.
The fact that no cell divisions were found in human material led to the conclusion that they should also be absent during wound healing. The fact that in studies of corneal endothelial wound healing, using scanning electron microscopic techniques, in cats and non-human primates did not actually reveal proliferative activity (Van Horn and Hyndiuk 1975) strengthened the impression that human corneal endothelial healing involved no proliferation, and that the two animal species studied should be suitable to imitate a human situation in experiments. The animals used were adults, even adult rabbits (Mills and Donn 1960; Oh 1963) show no spontaneous cell division in the corneal endothelium. The spontaneous cell divisions observed in young adult rabbits should be viewed in the light of yet-to-be-completed organ formation (Ham and Cormack 1979).
Whether spontaneous cell divisions occur in human neonates is unknown. The chance of finding proliferative activity is best when autoradiographic techniques are used because the S-phase lasts longer than the M-phase, and consequently the chance of finding a proliferating cell is better during the S-phase.
Studies involving the labelling of young human corneal endothelium in vitro,

in the manner described in this thesis, in order to establish whether proliferation occurs, have never been performed.

All tissues of an organism except nerve cells are subject to physiological regeneration. Particularly cells of tissue exposed to severe physiological demands are likely to have great powers of physiological regeneration. Examples are the cells of the skin, the intestinal epithelium and the bone marrow.

Compared with the epithelium of the skin, that of the cornea is very exposed to microtraumata. There is a constant threat of dehydration; there is exposure to marked differences in temperature and minute particles causing minute lesions; a high degree of physiological regeneration is therefore required and present.

The liver, whose cells are for the most part in the postmitotic phase (Cowdry 1942), shows much less physiological regeneration. The liver cell cycle can cover a period of several months. The demand for physiological regeneration of vascular endothelium is likewise low, and for corneal endothelium it is in fact extremely low, or even absent (absence of spontaneous cell divisions in specimens indicates this).

Corneal endothelium is in an anatomically privileged position and its task is 'merely' to form a barrier between stroma and anterior chamber. Unlike vascular endothelium, moreover, it is hardly exposed to microtraumata. These tissues -liver, vascular endothelium and corneal endothelium - are sometimes described as 'bradytropic' tissues in view of their low physiological regeneration. In view of their high metabolic activity, this designation is not entirely appropriate. However, these 'bradytropic' tissues are capable of rapid regeneration when the situation requires this. For example the liver shows a high degree of liver cell regeneration after subtotal hepatectomy (Jerusalem and Zaki 1958). Vascular endothelium shows a similar reaction after both mechanical and chemical traumata (Schwartz et al. 1978; Haudenschild and Schwartz 1979; Hess et al. 1982).

The present study (see subsection 4.2 and 4.3) has demonstrated that this rapid regeneration, migration and proliferation, occurs also in human corneal endothelium.

There are many similarities in the reaction pattern which follows damage to 'bradytropic' (liver, vascular endothelium, corneal endothelium) and that which follows damage to 'non-bradytropic' tissues (skin, corneal epithelium, intestinal epithelium). The cells which surround the wound and function as lining lose part of their junctions and arrange themselves more loosely (Ortonne et al. 1981). The first reaction is migration, which 24 hours after its start is supported by mitotic activity (bradytropic tissues) or by an increase in the normally present mitotic activity (non-bradytropic tissues). This proliferative activity attains its maximum after about 3 days, and then gradually returns to its initial level. This healing of skin wounds has been studied in

detail, and was found to depend on many parameters (Pollack 1979, 1979a, 1979b; Ortonne et al. 1981).
When the damage is not confined to the covering layer but also affects the underlying structure, a healing process starts in this tissue also (the subcutis of the skin, the vascular wall of the vascular endothelium, the stroma of the corneal endothelium). The vital cells migrate into the wound area and begin to show mitotic activity as well as to synthesize collagen. This proliferative process requires a longer starting time: about 5 days (Binder et al. 1980; Goder 1980; Pollack 1979). Once the covering layer closes the wound, granulation activity in the underlying wound diminishes, or even fails to start (Haudenschild and Schwartz 1979). This phenomenon was also observed in the present study (fig. 20).
Rapid re-endothelialization of the wound more or less guarantees ideal wound healing (Witmer 1980), and in this respect endothelial healing can be regarded as a race against time.
So far as corneal wounds are concerned, rapid re-endothelialization could arrest the granulation process in the stroma, thus preventing retrocorneal membrane formation. Moreover, rapid re-endothelialization can minimize a possible immunological acknowledgement of and reaction to the antigenic diffences of the cel membrane between regenerating and resting endothelium (Manski and Whitside 1974).
Due to their localization in the eyeball, corneal endothelium and lens epithelium are not in direct contact with the blood circulation. In this respect a few comparisons can be made between in-vivo and in-vitro studies. The epithelium of the lens, for example, shows relatively low proliferative activity in vivo. Isolated cells of the lens epithelium, however, show very high proliferative activity under tissue culture conditions. So high in fact that about 700 reduplications occur within a few weeks (Mungyer and Jerusalem 1979). It is the general experience of tissue culture specialists that proliferation of cells in vitro is preceded by a kind of adaptation period. During this period the seeded cells have to build up an ultimate culture condition which a cell culture requires in order to proliferate.

The minimum number of cells per millilitre of culture medium required to ensure this optimal micro-environment for the cells is 10^4 cells (Diamond et al. 1973) to 10^6 cells (Halle 1976). The anterior chamber with its volume of about 0.25 ml might be regarded as a kind of tissue culture chamber in which about 200,000-400,000 endothelial cells are kept in culture. Or, rather than as a tissue culture chamber, it could be described as a kind of perfusion chamber. The aqueous humour is produced in the ciliary body, nourishes the cornea and lens, and drains the metabolites off via the trabecular system. Aqueous humour production under normal conditions is estimated to amount to 2 μl/minute, i.e. 1% of the volume of the anterior chamber (Sears 1981).

This continuous refreshing of the 'medium' might be at odds with the creation of the micro-environment in which proliferation could take place. Consequently there can be no physiological regeneration to compensate the endothelial loss resulting from the ageing process. Apart from this 'medium' factor, contact inhibition of the endothelial cells also plays a role.

Whether the posttraumatic change in the composition of aqueous humour observed in in-vivo studies as stimulating endothelial proliferation (Kornblueth and Tenenbaum 1956; Radius et al. 1980) also occurs in the human situation, is unknown. Nor is it clear to which extent the diminution of contact inhibition observed in in-vivo human endothelial wound healing is responsible for the proliferative activity observed.

However, further research is required to identify, analyse and eliminate the factors which delay or even prevent corneal endothelial proliferation during wound healing. It seems logical that the effect of topical corticosteroids on corneal endothelial wound healing observed in rabbits (Polack and Rosen 1967; Sanchez and Polack 1974) also applies to human subjects. Whether the inhibitory effect on proliferative potency observed in rabbits involves a changed composition of aqueous humour or a direct effect on the endothelial cells, is unknown.

An effect of topical corticosteroids on human corneal endothelial wound healing may be of clinical importance, especially since it has been demonstrated in this study that proliferation is a feature of the wound healing process. Research will also have to be devoted to methods of stimulating the observed proliferation to ensure even more rapid re-endothelialization and promote ideal wound healing.

SUMMARY

Chapter 1 presents a general introduction and explains the problem statement which prompted this study

Chapter 2 discusses the embryology, anatomy and physiology of the cornea, wound healing in general, and corneal endothelial wound healing in particular. It focuses special attention on the anatomy of the cornea and the intercellular junctions of its endothelium The physiological function of the corneal endothelium is briefly explained. The cell cycle is discussed in order to elucidate the techniques used to study proliferation of a given type of tissue The processes involved in wound healing in general are discussed with reference to the healing of a cutaneous wound
Corneal endothelial wound healing has initially been studied mostly in animal experiments using rabbits as test animals In view of the evident proliferative properties of rabbit corneal endothelium, reports on subsequent experiments show a preference for test animals with endothelial characteristics that approximate those of human corneal endothelium as closely as possible. The change was made in accordance with the current view that proliferation of human corneal endothelium does not occur in wound healing Both cats and non-human primates seemed to be ideal test animals when it was demonstrated that the corneal endothelium in these species has virtually no more proliferative properties.
In the most recent literature there are indications that corneal endothelium in primates, including man, is capable of synthesizing DNA as a feature of wound healing (Svedberg 1975, Gloor et al 1980, Simonsen et al 1981) Mitotic as well as amitotic figures of cell division have been demonstrated during wound healing in response to growth factors (Fujikawa et al 1980) and also without growth factors (Zagorski 1080) in in-vitro human corneal endothelial wound healing

Chapter 3 describes the experimental set-up and the method of investigation in three different parts of the study
The first part, for which nine adult monkeys *(Macaca mulatta)* were available, concerned the localization and degree of endothelial loss after a penetrating corneal allograft This was studied morphologically with the aid of vital staining, scanning electron microscopy and cross-sections The wound healing after such a graft was studied by the same method
Next, the healing of a purely endothelial wound was studied for the prolifera-

tion, and a similar study was made of a penetrating corneal autograft
A flat specimen of the entire cornea was prepared Endothelial cell migration was studied with silver staining, and autoradiography was used to study the proliferative aspect The wound was exposed in vivo to tritium thymidine during one hour The DNA-synthesizing cells incorporated the label during this hour This method made it possible to study both migration and proliferation in the same specimen, even when a stromal wound was involved This combined technique, sometimes with nuclear staining added, was used in its entirety or in parts to study corneal endothelial wound healing in 152 human corneas in vitro This study was performed at the Bascom Palmer Eye Institute, Miami, Florida, USA, and was subdivided into nine experiments

The purpose of these experiments was to establish whether human corneal endothelium is capable of proliferation as a feature of wound healing, and the time at which this proliferative proces occurs The influence of exogenous factors on wound healing was also studied in these experiments, while other experiments were performed preparatory to the study of in-vivo human corneal endothelial wound healing The in-vivo human corneal endothelial wound healing was studied in the third part of the study
The latter made it necessary to induce the endothelial wound transcorneally with the aid of a cryo-probe, in contrast to the mechanical lesions studied in most of the in-vitro experiments The study of human corneas in vivo was likewise done at the Bascom Palmer Eye Institute, Miami, Florida, USA

Chapter 4 discusses the results of the experiments The in-vivo experiments with adult monkeys revealed that endothelial damage was mainly localized along the deep wound margins The results of vital staining and those of scanning electron microscopy were in agreement The areas of Descemet's membrane where endothelial cells had been lost as a result of the surgical trauma, were re-endothelialized within a few days
Re-endothelialization of the stromal wound, however, took much longer During a period of observation of maximally four weeks, re-endothelialization was found only at sites where good adaptation of the wound margins had existed In areas showing lack of re-endothelialization granulating tissue from the stromal wound
Combined silver staining and autoradiography revealed that, both in a purely endothelial wound and in corneal autografts, endothelial cell migration started first, to be followed by endothelial proliferation The latter was largely confined to the central part of the wound As soon as the endothelial cells established closer contact with each other, DNA synthesis (i e the proliferative component) diminished
The in-vitro experiments with 152 human corneas also showed that, after a corneal endothelial trauma, cell migration starts first, to be followed after a

latent period by proliferation. The age of the cornea donors ranged from 1 to 76 years.
The presence of autoradiographically positive mitotic figures demonstrated that DNA synthesis is followed by cell division. No qualitative difference was found between a mechanical wound and a transcorneally induced cryolesion of the endothelium. Nor was the quality of endothelial wound healing dependent on the presence of foetal calf serum in the culture medium.
The in-vivo study revealed that corneal endothelium is capable of proliferation as a feature of the wound healing process. This study was done in one 65-year-old and one 67-year-old patient, whose corneal wounds were studied 3 and 5 days, respectively, after infliction.

Chapter 5 discusses the importance of undisturbed wound healing to ensure optimal endothelial function after a corneal graft. Histopathological studies of failed corneal grafts nearly always show that deep-layer wound healing was disturbed, often leading to retrocorneal membrane formation. The current view that human corneal endothelium is incapable of proliferation is based on the fact that spontaneous cell division does not occur in it after completion of the growth phase of the eye.
Since corneal endothelial cell divisions do occur spontaneously in the corneal endothelium of young adult rabbits (4-6 months), before disappearing almost completely with increasing age, young adult rabbits are considered unsuitable as test animals in experiments imitating the human corneal graft situation. Monkeys and cats as well as adult rabbits are believed to be more suitable for this purpose.
The results of this study demonstrate that corneal endothelium is indeed capable of proliferation as a feature of wound healing, both in non-human primate corneas in vivo and human corneas in vitro and in vivo.
In human subjects this phenomenon was observed even at an advanced age. Proliferation reached a peak early in the course of wound healing (on the second and the third day) and gradually diminished, regardless of whether re-endothelialization was or was not completed.
The endothelial proliferation causes the cellular density in the wound area to increase, and this contributes to more rapid re-endothelialization. Re-endothelialization of the stromal wound has an inhibitory effect on the proliferative process, in the stroma. By ensuring good adaptation of the deep-layer wound (and rapid re-endothelialization) the stromal granulation process can be minimized and retrocorneal membrane formation prevented.
In rabbits, endothelial proliferation as a feature of wound healing is inhibited by topical corticosteroids. Further research will be required to establish whether this inhibition also occurs in human corneal endothelial wound healing. Especially since this study has demonstrated that human corneal endothelium is capable of proliferation as a feature of wound healing in vivo.

In de algemene inleiding, hoofdstuk 1, wordt de probleemstelling, die aanleiding was tot dit onderzoek uiteengezet. Hoofdstuk 2 behandelt de embryologie, anatomie, fysiologie van de cornea, wondheling in het algemeen en wondheling van het corneaendotheel in het bijzonder.
Met name wordt ingegaan op de anatomie van de cornea en de intercellulaire verbindingen van het endotheel. De fysiologische functie van het corneaendotheel wordt in het kort uiteengezet. Om de technieken, toegepast om proliferatie van een weefselsoort te onderzoeken, te verduidelijken, wordt in het kort ingegaan op de celcyclus. De processen die bij elke wondgenezing optreden, worden aan de hand van de genezing van een huidwond besproken.
De wondgenezing van het corneaendotheel is grotendeels gebaseerd op onderzoek, waarbij het konijn het proefdier was. Wegens het aanwezig zijn van proliferatieve eigenschappen van het konijnencorneaendotheel, wordt in latere literatuur de voorkeur gegeven aan proefdieren waarvan de endotheeleigenschappen zoveel mogelijk overeenkomen met die van de mens. Dit omdat volgens het huidige concept proliferatie van het menselijke corneaendotheel bij de wondgenezing niet voorkomt. Zowel de kat als de niet-humane primaat lijken het proefdier bij uitstek te zijn. Bij deze dieren is aangetoond, dat het corneaendotheel vrijwel geen proliferatieve eigenschappen meer bezit.
In de meest recente literatuur zijn er aanwijzingen dat het corneaendotheel van de primaat inclusief de mens in staat is DNA te synthetiseren als onderdeel van de wondgenezing (Gloor et al. 1980, Svedberg 1975, Simonsen et al. 1981). Onder invloed van groeistoffen zijn er zowel mitotische als amitotische delingsfiguren aangetoond tijdens de wondgenezing (Fujikawa et al. 1980), als ook zonder groeistoffen (Zagorski 1980) bij de humane corneaendotheelwondgenezing in vitro.
De proefopstelling en onderzoekmethode worden in hoofdstuk 3 beschreven. In het eerste deel van het onderzoek, waarin beschikt kon worden over 9 volwassen apen, wordt beschreven waar en in welke mate het endotheelverlies optreedt tijdens een allologe penetrerende corneatransplantatie. Dit werd morfologisch bestudeerd met vitale kleuring, scanning electronenmicroscopie en dwarscoupes. De wondgenezing die volgde na een dergelijke ingreep werd volgens dezelfde methode bestudeerd.
Vervolgens werd de endotheelwondgenezing van een zuivere endotheelwond onderzocht op het voorkomen van proliferatie, gevolgd door eenzelfde onderzoek bij een autologe penetrerende corneatransplantatie.
Er werd een endotheelvlakpreparaat gemaakt van de hele cornea. De endotheelmigratie werd onderzocht met zilverkleuring, terwijl het proliferatieve aspect werd onderzocht door middel van autoradiografie.

Tritium thymidine werd gedurende een uur in vivo in contact gebracht met de wond. Door de DNA synthetiserende cellen werd gedurende dit uur tritium thymidine geïncorporeerd. Met deze methode kon in hetzelfde preparaat zowel migratie als proliferatie onderzocht worden, ook wanneer er een stromale wond aanwezig was.
Deze gecombineerde techniek, uitgebreid met een kernkleuring, werd in zijn geheel of in onderdelen gebruikt bij de bestudering van de corneaendotheelwondgenezing van 152 humane corneas in vitro.
Dit onderdeel dat in het Bascom Palmer Eye Insitute, Miami, Florida, USA werd uitgevoerd, was onderverdeeld in 9 experimenten. De experimenten hadden tot doel na te gaan of het humane corneaendotheel in staat was tot proliferatie als onderdeel van de wondgenezing en wanneer dit proliferatieproces optrad in de tijd. Tevens werd in dit onderdeel de invloed van exogene factoren op de wondgenezing bestudeerd en werden experimenten uitgevoerd ter voorbereiding van het derde onderdeel van het onderzoek, namelijk bestudering van de corneaendotheelwondgenezing bij de mens in vivo.
Hiervoor was het nodig om de endotheelwond transcorneaal, door middel van bevriezing, aan te brengen, in tegenstelling tot de mechanische laesies die gebruikt werden in de meeste van de in vitro experimenten. Het in vivo onderdeel werd eveneens in het Bascom Palmer Eye Institute, Miami, Florida, USA, uitgevoerd.
In hoofdstuk 4 worden de resultaten van de proeven besproken. Uit de in vivo proeven met de volwassen apen bleek, dat de endotheelbeschadiging voornamelijk langs de diepe wondranden gelocaliseerd was. De resultaten van de vitale kleuring en de scanning electronenmicroscopie waren met elkaar in overeenstemming. De gebieden van de membraan van Descemet waar de endotheelcellen verloren waren gegaan door het operatietrauma, werden in enkele dagen weer bedekt.
De reëndothelialisatie van de stromawond duurde daarentegen veel langer, bij de maximale observatieperiode van 4 weken, was de reëndothelialisatie slechts op die plaatsen tot stand gekomen, waar een goede adaptatie van de wondranden aanwezig was. In die gebieden waar dat niet het geval was, werd de reëndothelialisatie belemmerd door granulerend wondweefsel vanuit het stroma.
De gecombineerde zilverkleuring en autoradiografie toonde aan, dat zowel bij een zuivere endotheelwond als bij de autologe corneatransplantatie, eerst het migratieproces van de endotheelcellen op gang kwam, om gevolgd te worden door proliferatie van endotheel.
De proliferatie vond voornamelijk plaats in het centrale deel van de wond. Zodra de endotheelcellen nauwer contact met elkaar kregen, nam de DNA synthese (dus de proliferatieve component) af.
Bij de in vitro experimenten met 152 humane corneas kon ook worden aangetoond, dat na een corneaendotheelwond eerst het migratiemechanisme

op gang komt, na een latentietijd gevolgd door proliferatie De donorleeftijd varieerde van 1 tot 76 jaar
Dat de DNA synthese gevolgd wordt door celdeling, werd door de aanwezigheid van autoradiografisch positieve mitosefiguren, aangetoond, Kwalitatief werd er geen verschil gevonden tussen een mechanisch en een transcorneal geinduceerde cryo-endotheelwond De endotheelwondgenezing was kwalitatief niet afhankelijk van foetaal kalfsserum
Het in vivo onderzoek toonde aan, dat het corneaendotheel in staat was tot proliferatie als onderdeel van de wondgenezing Dit onderzoek werd uitgevoerd bij een 65 en een 67 jarige patient De corneawond werd bestudeerd na respectievelijk 3 en 5 dagen na het aanbrengen van de wond
Histopathologisch onderzoek van mislukte corneatransplantaties toont vrijwel altijd aan, dat er stoornissen in de wondgenezing van de diepe lagen zijn geweest, vaak gepaard gaand met de vorming van retrocorneale membranen
Het belang van een ongestoorde genezing voor het verkrijgen van een optimale functie van het endotheel na een corneatransplantatie, wordt uiteengezet in hoofdstuk 5
Het huidige concept, dat het corneaendotheel van de mens niet in staat zou zijn tot proliferatie, berust op het feit, dat er spontaan in het endotheel van de cornea geen celdeling voorkomt, alleen tijdens de groeifase van het oog
Omdat bij het jong volwassen konijn (4-6 maanden) nog wel celdelingen van het corneaendotheel voorkomen, hoewel dit vrijwel volledig verdwijnt bij het ouder worden, wordt het jong volwassen konijn niet geschikt geacht als proefdier om humane corneatransplantaties na te bootsen De kat en de aap zouden hier beter voor geschikt zijn, evenals het volwassen konijn
De resultaten van dit onderzoek tonen aan, dat zowel bij de aap in vivo als bij de mens in vitro en in vivo, het endotheel van de cornea in staat is tot proliferatie als onderdeel van de wondgenezing
De piek in de proliferatie werd vroeg in de wondgenezing gevonden, op de tweede en derde dag Dit nam geleidelijk af, onafhankelijk van het feit of de reendothelialisatie voltooid was of niet
Door de proliferatie van het endotheel neemt de celdichtheid in het wondgebied toe, welke bijdraagt tot een snellere reendothelialisatie Endotheelbedekking van de stromawond heeft een remmende invloed op het proliferatieproces in het stroma Door voor een goede wondadaptatie van de diepe lagen te zorgen kan door snelle reendothelialisatie het stromale granulatieproces tot een minimum beperkt blijven en daarmee soms retrocorneale membraanvorming voorkomen worden
Endotheelproliferatie als onderdeel van de wondgenezing bij het konijn wordt geremd door locale toediening van corticosteroiden Nu is aangetoond dat het humane corneaendotheel tot proliferatie als deel van de wondgenezing in vivo in staat is, zal nader onderzoek nodig zijn om na te gaan of deze remming ook bij de mens optreedt

BAUM, J L AND SILBERT, A M: *Aspects of corneal woundhealing in health and disease.* Trans. Ophthalmol Soc. U K. **98:** 348-351, 1978.

BAUM, J L, NIEDRA, R, DAVIS, C AND YUE, B Y J T: *Mass culture of human corneal endothelial cells* Arch Ophthalmol **97:** 1136-1140, 1979.

BINDER, R F AND BINDER, H F: *Regenerative processes in the endothelium of the cornea.* Arch Ophthalmol **57:** 11-13, 1957

BINDER, P S, WICKHAM, M G, ZAVALA, E Y AND AKERS, P H. *Corneal anatomy and woundhealing;* in: 'Symposium on medical and surgical diseases of the cornea' (Edited by Barraquer J I, Binder, P S., Buxton, J.N., Fine, M., Jones, D.B, Laibson, P R., Nesburn, A B., Paton, D, Troutman, R.C., The C V. Mosby Compagny, St. Louis, pp. 1-35, 1980).

BITO, L Z AND HARDING, C V: *Tritium retention by corneal endothelium after incorporation of H^3-Thymidine.* Arch Ophthalmol **65:** 553-556, 1961

BLOOM, W AND FAWCETT, D W: *A textbook of histology 10th ed.* (W B. Saunders, Comp. Philadelphia, pp. 83, 84, 386, 1975).

BOURNE, W M AND KAUFMAN, H E *Specular microscopy of human corneal endothelium in vivo.* Am. J. Ophthalmol. **81:** 319-323, 1976

BOURNE, W M, MCCAREY, B E AND KAUFMAN, H E: *Clinical specular microscopy.* Trans. Am. Ophtalmol Otolaryn. **81:** 743-753, 1976.

BROWN, S I AND KITANO, S. *Pathogenesis of the retrocorneal membrane.* Arch. Ophthalmol. **75:** 518-525, 1966.

BRYANT, B J: *Reutilization of leukocyte DNA by cells of regenerating liver.* Exptl. Cell. Res. **27:** 70-79, 1962.

BURNS, R R, BOURNE, W M AND BRUBAKER, R F *Endothelial function in patients with cornea guttata.* Invest Ophthalmol. Vis Sci **20:** 77-85, 1981.

CAPELLA, J A: *Regeneration of endothelium in diseases and injured corneas.* Am. J. Ophthalmol. **74:** 810-817, 1972.

CARRERAS, B y JORDANO, J: *Lesions en al endotelio corneal consecutivas a crioterapia* Ann. Inst. Barraquer **12:** 147-161, 1974-75

CHI, H H AND KEIMAN, C D. *Effects of freezing on ocular tissues: I. Clinical and histologic study of corneal endothelium* Am J. Ophthalmol **61:** 630-641, 1966

CHI, H H, TING, C C AND KATZIN, H M *Healing process in the mechanical denudation of the corneal endothelium* Am. J. Ophthalmol **49:** 693-703, 1960.

CHI, H H, TING, C C AND KATZIN, H M *Histopathology of corneal endothelium.* Am. J. Ophthalmol. **53:** 215-234, 1962.

CLAEVER, J E: *Thymidine metabolism and cellkinetics.* (Wiley New York, 1967).

COGAN, D: *Studies on the clinical physiology of the cornea.* Am. J. Ophthalmol. **32:** 625-633, 1949.

COULOMBRE, A J: *Regulation of ocular morphogenesis,* in: 'Symposium on embryonic development' Invest. Ophthalmol. **8:** 25-31, 1969

COWDRY, E V: *Problems of aging.* (William & Wilkins 1942).

DIAMOND, L, MCFALL, R, TASHIRO, J AND SABATINI, D: *The WIRL-3 rat liver cells lines and their transformed derivates* Cancer Research **33:** 2627-2636, 1973.

DIDERHOLM, H, FICHTELIUS, K E AND LINDER, O: *Availability time of ^{3}H-label after administration of ^{3}H-Thymidine in vivo.* Exptl. Cell. Res. **27:** 431-435, 1962.

DOUGHMAN, D J, VAN HORN, D L, RODMAN, W P, BYRNES, P AND LINDSTROM, R L: *Human corneal endothelial layer repair during organ culture.* Arch. Ophthalmol **94:** 1791-1796, 1976.

DÜBLIN, I *Vergleichend-embryologische Untersuchungen über die Frühentwicklung der Hornhaut und der Pupillarmembran bei Reptilien, Vogeln und Saugern.* Acta Anat. **76:** 381-408, 1970.

DUKE ELDER, S AND COOK, C *Normal and abnormal development;* in System of Ophthalmology vol. III, part I. Embryology. (Edited by Duke Elder, S and Cook, C., Henry Kimpton, London, pp 41-42, 164-170, 1963)

DULBECCO, R *Topoinhibition and serum requirement of transformed and untransformed cells* Nature **227:** 802-806, 1970

EVERETT AND TROUTMAN, R C *Placement of sutures used in corneal incisions* Am J Ophthalmol **82:** 786-789, 1976

EWER, M S *Zur Fruhentwicklung des Stroma Corneae und der Pupillar membran beim Menschen* Acta Anat **75:** 37-46, 1970

FABRICANT, R N, ALPAR, A J, CENTIFANTO, Y M AND KAUFMAN, H E *Epidermal growthfactor receptors on corneal endothelium* Arch Ophthalmol **99:** 305-308, 1981

FARQUHAR, M G AND PALADI, G E *Junctional complexes in various epithelia* J Cell Biol **17:** 375-412, 1963

FAURE, J P, KIM, Y Z AND GRAF, B *Formation of giant cells in the corneal endothelium during regeneration after destruction by freezing* Exp Eye Res **12:** 6-12, 1971

FINE, B S AND YANOFF, M *Ocular histology a text and atlas,* (Harper & Row, Publishers, New York, pp 33-36, 163-193, 261-270, 1979)

FOLKMAN, J AND MOSCONA, A *Role of cell shape in growth control* Nature **273:** 345-349, 1978

FUJIKAWA, L S, WICKHAM, M G AND BINDER, P S *Wound healing in cultured corneal endothelial cells* Invest Ophthalmol Vis Sci **19:** 793-801, 1980

GELFANT, S *Inhibition of celldivision A critical and experimental analysis* Internat Rev Cytolog **14:** 1-39, 1963

GLOOR, B P, GLOOR, M L, MARSHALL, J AND MESZAROS, J *Healing of mechanical wounds of the corneal endothelium of rhesus monkeys and rabbits,* in 'The cornea in Health and Disease, VIth Congress of the European Society of Ophthalmology, Brighton 21-25 april, 1980' (Edited by Trevor-Roper, P, Academic Press Inc, London, pp 97-102, 1981)

GODER, G *Zur Wundheilung nach Keratoplastik,* in 'Wundheilung des Auges und ihre Komplikationen' (Edited by Naumann G O H und Gloor, B P, J F Bergmann Verlag, Munchen, pp 195-198, 1980)

GOLDMAN, J N AND BENEDEK, G B *The relationship between morphology and transparancy in the nonswelling corneal stroma of the shark* Invest Ophthalmol **6:** 574-600, 1968

GORDON, S R AND ROTHSTEIN, H *Studies on corneal endothelial growth and repair I Microfluorometric and autoradiographic analysis of DNA synthesis, mitosis and amitosis following freeze injury* Metab Ophthalmol **2:** 57-63, 1978

GOSPODAROWICZ, D AND GREENBURG, G *The effect of epidermal and fibroblast growthfactors on the repair of corneal endothelial wounds in bovine corneas maintained in organ culture* Exp Eye Res **28:** 147-157, 1979

GOSPODAROWICZ, D AND ILL, C *The extracellular matrix and the control of proliferation of corneal endothelial and lens epithelial cells* Exp Eye Res **31:** 181-199, 1980

GOSPODAROWICZ, D GREENBURG, G AND ALVARADO, J *Transplantation of cultured bovine corneal endothelial cells to species with nonregenerative endothelium* Arch Ophthalmol **97:** 2163-2169, 1979

GRABNER, G AND BRENNER, W *Unscheduled DNA repair in the human cornea following solar simulating radiation* Acta Ophthalmol **59:** 847-853, 1981

GRAY, P, PICKEL, F M, MASLR, M D AND HAYWEISER, L J *Oxazine dyes I Celestine blue Bwithiron as a nuclear stain* Stain Tech **31:** 141-150, 1956

GRAYSON, M *Diseases of the cornea* (The C V Mosby Compagny, London, pp 1-14, 1979)

HAHERKAMP, O *Allgemeine Pathologie der Wundheilung,* in 'Wundheilung des Auges und Ihre Komplikationen' (Edited by Naumann, G O H und Gloor, B P, J F Bergmann Verlag, München, pp 11-20, 1980)

HALFS, R H AND SPENCER, W H *Unsuccessful penetrating keratoplasties* Arch Ophthalmol **70:** 805-810, 1963

HALLE, W *Zell- und Gewebezuchtung bei Tieren* (VEB Guster Fischer Verlag Jena 1976)

HAM, A W AND CORMACK, D H *Histology 8th ed* (J B Lippincott Compagny, Philadelphia, pp 35-84, 194-201, 529-531, 1979)

HARRIS, J *Transport of fluid from the cornea* in 'Transparancy of the cornea' (Edited by Duke Elder S and Perkins E S, Blackwell Scientific Publications, Oxford, pp 73-86, 1960)

HAUDENSCHILD, C C AND SCHWARTZ, S W *Endothelial regeneration II Restitution of endothelial continuity* Lab Invest **41**: 407-418, 1979

HAY, E D AND DODSON, J W *Secretion of collagen by corneal epithelium I Morphology of the collagenous products produced by isolated epithelia grown on frozen killed lens* J Cell Biol **57**: 190-213, 1973

HELFER, M D, IRVINE, S R, STRAATSMA, B R AND FOOS, R Y *Wound healing after cataract extraction and position of the vitreous in aphakic eyes as studied postmortem* Tr Am Ophthalmol Soc **69**: 245-262, 1971

HESS, F, JERUSALEM, C, BRAUN, B AND ZELDER, O *A new experimental non-woven small caliber vascular prosthesis Preliminary observations on the re-endothelialisation process,* in 'Proc 6th Int Congres Microsurgery Soc Sydney 1981' (In Press)

HINZPETER, E N AND NAUMANN, G O H *Transplantation of the cornea in man and animal,* in 'Handbuch der allgemeinen Pathologie Achter Teil, Sechster Band' (Springer-Verlag, New York, pp 403-438, 1977)

HIRSCH, M, FAURE, J P, MARQUET, O AND PAYRAU, P *Regeneration de l'endothelium cornéen chez le lapin etude microscopique et relation avec l'épaisseur de la cornee* Arch Ophthalmol (Paris) **35**: 269-278, 1975

HIRSCH, M, RENARD, G, FAURE, J P AND POULIQUEN, Y *Formation of intercellular spaces and junctions in regenerating rabbit corneal endothelium* Exp Eye Res **23**: 385-397, 1976

HIRSCH, M, RENARD, G, FAURE, J P AND POULIQUEN, Y *Study of the ultrastructure of the rabbit corneal endothelium by the freeze-fracture technique apical and lateral junctions* Exp Eye Res **25**: 227-288, 1977

HODSON, S *The endothelial pump of the cornea* Invest Ophthalmol **16**: 589-591, 1977

HOGAN, M J, ALVARADO, J A AND WEDDELL, J E *Histology of the human eye* (W B Saunders Co, Philadelphia, pp 55-111, 1971)

HONNEGGER, H UND SCHIERHÖLTER, R *Das Histologische Bild des Hornhautendothels im Hautchenpraparat* Alb v Graefes Archiv fur Ophthalmologie **166**: 359-377, 1963

HUGHES, W L, BOND, V L, BRECHER, G, CRONKITE, E P, PAINTER, R B, QUASTLER, H AND SHERMAN, F G *Cellular proliferation in the mouse as revealed by autoradiography with tritiated thymidine* Proc N A S **44**: 476-483, 1958

INOMATA, H, SMELSER, G K AND POLACK, F M *Fine structure of regenerating endothelium and Descemet's membrane in normal and rejecting corneal grafts* Am J Ophthalmol **70**: 48-64, 1970

IWAMOTO, T AND SMELSER, G K *Electron microscopy of the human corneal endothelium with reference to transport mechanisms* Invest Ophthalmol **4**: 270-284, 1965

JAKUS, M A *The fine structure of the human cornea,* in 'The structure of the eye' (Edited by Smelser, G K, Academic Press, New York, pp 343-366, 1961)

JAKUS, M A *Ocular fine structure Selected electron micrographs* (Little Brown, Boston, 1964)

JERUSALEM, C UND ZAKI, F G *Zur Frage der Mitose-Induktion in der Rattenleber* Zeitschrift fur mikroskopisch-anatomische Forschung **64**: 321-363, 1958

JOHNSTON, M C, NODEN, D M, HAZELTON R D, COULOMBRE, J I AND COULOMBRE, A J *Origins of avian ocular and periocular tissues* Exp Eye Res **29**: 27-43, 1979

KANAI, A, MUSTAKALLIO, A H AND KAUFMAN, H E *Electron microscopic studies of corneal endothelium The abnormal endothelium associated with retrocorneal membrane* Ann Ophthalmol **4**: 564-576, 1972

KARA-JOSE, N, LORENZETTI, D W C, MCALLIFFE, R AND CONTI, T *Time response effects of corticosteroids on corneal wound healing* Can J Ophthalmol **7**: 48-51, 1972

KARP, G *Cell Biology* (Mc-Graw Hill Inc New York, pp 282-293, 639-731, 1979)

KAUFMAN, H E AND KATZ, J I *Pathology of the corneal endothelium* Invest Ophthalmol Vis Sci **16**: 265-268, 1977

KAUFMAN, H E, CAPELLA, J A AND ROBBINS, J E *The human corneal endothelium Am J Ophthalmol* **61**: 835-841, 1966

KAYE, G I AND PAPPAS, G D *Studies on the cornea I The fine structure of the rabbit cornea and the uptake and transport of colloidal particles by the cornea in vivo* J Cell Biol **12:** 457-479, 1962

KAYE, G I, PAPPAS, G D, DONN, A AND MALIET, N *Studies on the cornea II The uptake and transport of colloidal particles by the living rabbit cornea in vitro* J Cell Biol **12:** 481-501, 1962

KAYE, G I, MISHIMA, S, COLE, J D AND KAYE, N W *Studies on the cornea VII Effects of perfusion with a Ca++ free medium on the corneal endothelium* Invest Ophthalmol **7:** 53-66, 1968

KAYI, G I, SIBLEY, R C AND HOEFLE, F B *Recent studies on the nature and function of the corneal endothelial barrier* Exp Eye Res **15:** 585-613, 1973

KAYE, G I, HOEFLE, F B AND DONN, A *Studies on the cornea VIII Reversibility of the effects of in vitro perfusion of the rabbit corneal endothelium with calcium free medium* Invest Ophthalmol **12:** 98-113, 1973

KENYON, K R, STARK, W J AND STONE, D L *Corneal endothelial degeneration and fibrous proliferation after pars plana vitrectomy* Am J Ophthalmol **81:** 486-490, 1976

KHODADOUST, A A AND GREEN, K *Physiological function of regenerating endothelium* Invest Ophthalmol **15:** 96-101, 1976

KNÖBEL, H AND V DOMARUS, D *Zur Bildung der retrokornealen Membran nach perforierender Keratoplastik* Klin Mbl Augenheilk **175:** 191-196, 1979

KORNBLUTH, W AND TENENBAUM, E *The inhibitory effect of aqueous humor on the growth of cells in tissue culture* Am J Ophthalmol **42:** 70-74, 1956

KREUTZIGER, G O *Lateral membrane morphology and gap junction structure in rabbit corneal endothelium* Exp Eye Res **23:** 285-293, 1976

KURZ, G H AND D'AMICO, R *Histopathology of corneal graft failures* Am J Ophthalmol **66:** 184-199, 1968

KUWABARA, T *Current concepts in anatomy and histology of the cornea* Contact IOL Med J **4:** 101-131, 1978

LAHAV, M AND CADET, J C *Subepithelial fibrous tissue in failed corneal grafts, A histopathologic study* Albrecht v Graefes Arch klin Ophthalmol **211:** 145-154, 1979

LAING, R A, SANDSTROM, M M, BERROSPI, A R AND LEIBOWITZ, H M *Changes in the corneal endothelium as a function of age* Exp Eye Res **22:** 587-594, 1976

LANDHOLT, E *Die Wundheilung nach Kataraktoperation aus pathologisch-anatomischer Sicht,* in 'Wundheilung des Auges und ihre Komplikationen' (Edited by Naumann G O H und Gloor B P, J F Bergmann Verlag, Munchen, pp 275-277, 1980)

LEBLOND, C P, MESSIER, B AND KOPRIWA, B *Thymidine-^{3}H as a tool for the investigation of the renewal of cell populations* Lab Invest **8:** 296-308, 1959

LEMP, M A *Cornea and sclera* Arch Ophthalmol **94:** 473-490, 1976

LEUCHTENBERGER, C, LEUCHTENBERGER, R AND DAVIS, A M *A microspectrophotometric study of the desoxyribose nucleic acid (DNA) content in cells of normal and malignant human tissues* Am J Pathol **30:** 65-85, 1954

LEUENBERGER, P M, GNÄDINGER, M C AND CABERNARD, F *Zur Frage der Kollagensynthese durch corneales Epithel I Elektronen mikroskopische Studie an regeneriertem Epithel der Kaninchenhornhaut* Albrecht von Graefes Arch klin Ophthalmol **87:** 171-182, 1973

LOEWENSTEIN, W R *Cellular Communication by permeable Membrane Junctions,* in 'Cell Membranes' (Edited by Weismann, G and Clairborne, R, H P Publishing Co Inc, New York, pp 105-114, 1975)

LOEWENSTEIN, W R *The cell to cell membrane channel in development and growth,* in 'Differentiation and development' (Edited by Ahmad, F, Russel, J R, Schultz, J and Werner, R, Miami Winter Symposia 1978, Academic Press, New York, pp 399-409, 1978)

LOEWENSTEIN, W R AND PENN, R D *Intercellular communication and tissue growth II Tissue regeneration* J Cell Biol **33:** 235-242, 1967

MAENZA, R M AND HARDING, C V *Disappearance of tritiated thymidine and tritiated water from the anterior chamber of the rabbit eye* Nature **196:** 786-787, 1962

MANN, I *The development of the human eye* (Grune & Stratton Inc New York, 1950)

MANSKI, W AND WHITSIDE, T L *Cell-surface receptors of normal, regenerating, and cultured corneal epithelial and endothelial cells* Invest Ophthalmol **13:** 935-944, 1974

MARSHALL, J AND GRINDLE, C F J *Fine structure of the cornea and its development* Trans Ophthalmol Soc U K **98:** 320-328, 1978

MARTZ, E AND STEINBERG, M S *The role of cell-cell contact in 'contact' inhibition of cell division A review and new evidence* J Cell Physiol **79:** 189-210 1972

MATSUDA, H AND SMELSER, G K *Endothelial cells in alkali burned corneas ultrastructural alterations* Arch Ophthalmol **89:** 402-409, 1973

MATSUDA, H AND SMELSER, G K *Electron microscopy of corneal wound healing* Exp Eye Res **16:** 427-442, 1973a

MAURICE, D M *The structure and transparancy of the cornea* J Physiol **136:** 263-286, 1957

MAURICE, D M *The physics of corneal transparancy*, in 'Transparancy of the cornea' (Edited by Duke Elder S and Perkins E S , Blackwell Scientific Publications, Oxford pp 41-49 1960)

MAURICE, D M *Cellular membrane activity in the corneal endothelium of the intact eye* Experienta **24:** 1094-1095, 1968

MAURICE, D M *The location of the fluid pump in the cornea* J Physiol **221:** 43-54, 1972

MAURICE, D M AND GIARDINI, A *Swelling of the cornea in vivo after destruction of its limiting layers* Br J Ophthalmol **35:** 791-797, 1951

MCMANNUS, J F A AND MOWRY, R W *Staining methods, histologic and histochemical* (Paul B Hoeber Inc New York, pp 76, 77, 1960)

MCTIGUE, J W *The human cornea A light and electron microscopic study of the normal cornea and its alterations in various dystrophies* Tr Am Ophthalmol Soc **65:** 591-660, 1967

MEIER, S *Initiation of corneal differentiation prior to cornea-lens association* Cell Tissue Res **184:** 255-268, 1977

MICHELS, R G , KENYON, K R AND MAUMENEE, A E *Retrocorneal fibrous membrane* Invest Ophthalmol **11:** 822-831, 1972

MILLS, N L AND DONN, A *Incorporation of tritium labeled thymidine by rabbit endothelium* Arch Ophthalmol **64:** 443-446, 1960

MISHIMA, S *In vivo determination of fluorescein permeability of the corneal endothelium* Arch Ophthalmol (Paris) **35:** 191-196 1975

MISHIMA, S *Clinical investigations on the corneal endothelium XXXVIII Edward Jackson Memorial Lecture* Am J Ophthalmol **93:** 1-129, 1982

MISHIMA, S AND MAURICE, D M *The effect of normal evaporation on the eye* Exp Eye Res **1:** 46-52, 1961

MISHIMA, S AND HEDBYS, B P *The permeability of corneal epithelium and endothelium to water* Exp Eye Res **6:** 10-32, 1967

MOORE, T E AND ARONSON, S B *Role of surgical factors in corneal graft failure* in 'Corneal Graft Failure Ciba Foundation Symposium No 15' (Associated Scientific Publishers, Amsterdam, pp 209-217, 1973)

MORTON, P L, ORMSBY, H L AND BASU , P K *Healing of endothelium and Descemet's membrane of rabbit cornea* Am J Ophthalmol **46:** 62-67, 1958

MUIR, A R AND PETERS, A *Quintuple layered membrane junctions at terminal bars between endothelial cells* J Cell Biol **12:** 443-448, 1962

MUNGYER, G AND JERUSALEM, R *Establishment of cultures from bovine lens epithelium* Tissue culture association manual **5:** 1177-1179 1979

NICHOLAS, J P *Whole mounts of the corneal endothelium* Arch Ophthalmol **73:** 555-558, 1965

O'DAY, D M , DANIEL, G AND SHERRARD, E S *Preparation of full thickness flat mounts of rabbit cornea A new approach* Brit J Ophthalmol **57:** 650-653, 1973

OH, J O *Changes with age in the corneal endothelium of normal rabbits* Acta Ophthalmol **41:** 568-573, 1963

OLSEN, T *The endothelial cell density after cataract surgery in young patients* Acta Ophthalmol **59:** 242-246 1981

OLSON, L E, MARSHALL, J , RICE, N S C AND ANDREWS, R *Effect of ultrasound on the corneal endothelium II The endothelial repair process* Brit J Ophthalmol **62:** 145-154, 1978

ORTONNE, J P , LÖNING, T , SCHMITT, D AND THIVOLET, J *Immunomorphological and ultrastructural aspects of keratinocyte migration in epidermal wound healing* Virchows Arch (Pathol Anat) **392:** 217-230, 1981

OTA, Y: *Endothelial permeability to fluorescein in corneal grafts and bullous keratopathy.* Jpn. J. Ophthalmol. **19:** 286-295, 1975

OLTERSEN, OP AND VEGGE, T: *Ultrastructure and distribution of intercellular junctions in corneal endothelium.* Acta Ophthalmol **55:** 69-78, 1977.

OVERTON, J: *Cell junctions and their development;* in. 'Progress in Surface and Membrane Science' (Edited by Cadenhead, D.A., Danielli, J.F. and Rosenberg, M.D., Academic Press, New York, pp. 161-208, 1974)

OZANICS, V. RAYBORN, M AND SAGUN, D: *Observations on the morphology of the developing primate cornea: Epithelium, its innervation and anterior stroma.* J. Morph. **153:** 263-298, 1977.

PAPPAS, GD: *Junctions between cells;* in: 'Cell Membranes' (Edited by Weismann, G. and Clairborne, R., H.P. Publishing Co. Inc., New York, pp. 87-94, 1975).

PEARCE, WG, TRIPATHI, RC AND MORGAN, C: *Congenital endothelial dystrophy. Clinical, pathological and genetic study.* Br. J. Ophthalmol. **53:** 577-591, 1969.

PEARSON, AA: *The development of the eye.* (American Academy of Ophthalmology and Otolaryngology, 1975).

PELC, SR AND VIOLA-MAGNI, MP: *III Decrease of labeled DNA in cells of the adrenal medulla after intermittent exposure to cold.* J. Cell Biol. **42:** 460-468, 1969.

POLACK, FM: *The corneal host-graft junction. Physiopathology of the scar.* Arch. Ophthalmol. (Paris) **35:** 139-152, 1975.

POLACK, FM: *The healing of corneal grafts;* in: 'Corneal Transplantation' (Grune & Stratton, Inc. New York, pp. 45-69, 1977).

POLACK, FM AND ROSEN, PN: *Topical steroids and tritiated thymidine uptake.* Arch. Ophthalmol. **77:** 400-404, 1967.

POLACK, FM, SMELSER, GK AND ROSE, J: *Long-term survival of isotopically labeled stromal and endothelial cells in corneal homografts.* Am. J. Ophthalmol. **57:** 67-78, 1964

POLLACK, SV: *Wound healing· A Review. I. The biology of wound healing.* J. Dermatol. Surg. Oncol. **5:** 389-393, 1979.

POLLACK, SV: *Wound healing: A Review. II. Environmental factors affecting wound healing.* J. Dermatol. Surg. Oncol. **5:** 477-481, 1979.

POLLACK, SV: *Wound healing: A Review. III. Nutritional factors affecting wound healing.* J. Dermatol. Surg. Oncol. **5:** 615-619, 1979.

POTTER, VR: *Metabolic products formed from thymidine;* in: 'The kinetics of cell proliferation' (Edited by Stohlman, F., Grune & Stratton, New York, pp. 104-110, 1959).

PRESCOTT, DM: *Reproduction of eukaryotic cells.* (Academic Press, New York, pp. 52, 131, 134, 1976).

RADIUS, RL, HERSCHLER, J, CLAFLIN, A AND FIORENTINO, G: *Aqueous humor changes after experimental filtering surgery.* A.M. J. Ophthalmol **89:** 250-254, 1980.

RAO, GN, SHAW, EL, ARTHUR, E AND AQUAVELLA, JV: *Morphological appearance of the healing corneal endothelium.* Arch. Ophthalmol. **96:** 2027-2030, 1978.

RAO, GN, STEVENS, RE, MANDELBERG, AI AND AQUAVELLA, JV: *Morphologic variations in graft endothelium.* Arch. Ophthalmol. **98:** 1403-1406, 1980.

RASMUSSEN, RF AND PAINTER, RB: *Radiation-stimulated DNA synthesis in cultured mammalian cells.* J. Cell Biol. **29:** 11-19, 1966.

REIM, WS AND SPANGLER, SG: *A new theory for the mechanism by which the endothelium controls corneal hydration.* Ala. J. Med. Sci. **13:** 196-205, 1976.

RENARD, G, HIRSCH, M, FAURE, JP, USUI, M AND POLLIQUEN, Y: *Ultrastructural study on the regeneration of the corneal endothelial cells.* Jap. J. Ophthalmol. **20:** 243-256, 1976.

RENARD, G, POULIQUEN, Y AND HIRSCH, M: *Regeneration of the human corneal endothelium.* Alb. v. Graefes Arch. klin. Ophthalmol. **215:** 341-348, 1981.

ROBBINS, JE, CAPELLA, JA AND KAUFMAN, HE: *A study of endothelium in keratoplasty and corneal preservation.* Arch Ophthalmol. **73:** 242-247, 1965.

RUBINI, JR, CRONKITE, E, BOND, V AND FLIEDNER, TM: *The metabolism and fate of tritiated thymidine.* J. Clin. Invest. **39:** 909-918, 1960.

RUDOLPH, R: *Inhibition of myofibroblasts by skin grafts.* Plastic and Reconstructive Surgery **63:** 473-480, 1979.

RUUSUVAARA, P *Endothelial cell densities in donor and recipient tissue after keratoplasty* Acta Ophthalmologica **58:** 267-277, 1980

RYAN, G B, CLIFF, W J, GABBIANI, G, IRLÉ, C, MONTANDON, D, STATKOV, P R AND MAJNO, G *Myofibroblasts in human granulation tissue* Human Pathology **5:** 55-67, 1974

RYCROFT, P V *Corneal graft membranes* Trans Ophthalmol Soc U K **85:** 317-325, 1965

SANCHEZ, J AND POLACK, F M *Effect of topical steroids on the healing of corneal endothelium* Invest Ophthalmol **13:** 17-22, 1974

SANCHEZ, J AND POLACK, F M *Autoradiographic study of retrocorneal membranes* Ann Ophthalmol **10:** 1547-1552, 1978

SANCHEZ, J, POLACK, F M, EVE, F R AND TROUTMAN, R C *Microsurgical sutures II Corneal endothelial healing and posterior wound closure after 'through and through' suturing* Can J Ophthalmol **9:** 48-55, 1974

SATO, T *Studies on the endothelium of the corneal graft* Jpn J Ophthalmol **22:** 114-126, 1978

SCHIMMELPFENNIG, B *Some aspects of human corneal endothelium viability* (Cornea preservation and cornea transplantation Amsterdam, jan 16, 1982)

SCHUTTEN, W H, VAN HORN, D L, BADE, B J AND FACULJAK, M L *Corneal endothelial autoradiography with the scanning electron microscope* Invest Ophthalmol Vis Sci **19:** 417-420, 1980

SCHUTZ, L AND MORA, P T *Growth of mouse fibroblasts in the presence of irradiated and lyophilized monolayers of the same type of cells* J Cell Physiol **71:** 7-16, 1968

SCHWARTZ, S M, HAUDENSCHILD, C C AND EDDY, F M *Endothelial regeneration I Quantitative analysis of initial stages of endothelial regeneration in rat aortic intima* Lab Invest **38:** 568-580, 1978

SEARS, M L *The aqueous,* in 'Adlers' Physiology of the eye, seventh ed '(Edited by Moses, R A , The C V Mosby Comp St Louis, pp 204-226, 1981)

SETÀLÀ, K *Response of human corneal endothelial cell to increased intraocular pressure A specular microscopic study* Acta Ophthalmologica 1980 **supplement 144 TO.**

SHERRARD, E S *Further studies on the retrocorneal membrane endothelium relationship* Br J Ophthalmol **53:** 808-818, 1969

SHERRARD, E S *The corneal endothelium in vivo Its response to mild trauma* Exp Eye Res **22:** 347-357, 1976

SHERRARD, E S AND RYCROFT, P V *Retrocorneal membranes I Their origin and structure* Br J Ophthalmol **51:** 379-386, 1967

SHERRARD, E S AND RYCROFT, P V *Retrocorneal membranes II Factors influencing their growth* Br J Ophthalmol **51:** 387-393, 1967[a]

SHOLLEY, M M, GIMBRONE JR, M A, AND COTRAN, R S *Cellular migration and replication in endothelial regeneration A study using irradiated endothelial cultures* Lab Invest **36:** 18-25, 1977

SILBERT, A M AND BAUM, J L *Origin of the retrocorneal membrane in the rabbit* Arch Ophthalmol **97:** 1141-1143, 1979

SIMONSEN, A H, SØRENSEN, K E AND SPERLING, S *Thymidine incorporation by human corneal endothelium during organ culture* Acta Ophthalmologica **59:** 110-118, 1981

SMELSER, G K AND OZANICS, V *Reaction of the cornea to injury and wound healing,* in 'Symposium on the cornea' (Edited by Castroviejo R , DeVoe, A G , Dohlman, C H , Elliot, J H , Fine, M and Smelser, G K , The C V Mosby Comp , St Louis, pp 121-131, 1972)

SMITH, E L *A review of retrograft membranes* Can J Ophthalmol **5:** 127-135, 1970

SMITH, R S, SMITH, L A, RICH, L AND WEIMAR, V *Effects of growth factors on corneal wound healing* Invest Ophthalmol Vis Sci **20:** 222-229, 1981

SMOLIN, G *A technique for staining and separating corneal endothelium* Am J Ophthalmol **65:** 232-236, 1968

SOMMERVILLE, G M AND SHEA, M *Effect of freezing on the corneal endothelium* Can J Ophthalmol **1:** 183-189, 1966

SOONG, H, FISCHBARG, J AND IWAMOTO, T *A method for preparing a viable endothelial layer, completely denuded of overlying stroma* Invest Ophthalmol Vis Sci **17:** 1113-1117, 1978

SPERLING, S *Early morphological changes in organ cultured human corneal endothelium* Acta Ophthalmologica **56:** 785-792, 1978

SQUIRES, E L AND WEIMAR, V L *Stimulation of repair of human corneal endothelium in organ culture by mesodermal growth factor* Arch Ophthalmol **98:** 1462-1466, 1980

STAATZ, W D AND VAN HORN, D L *The effects of aging and inflammation on corneal endothelial cells in rabbits* Invest Ophthalmol Vis Sci **19:** 983-986, 1980

STOCKER F W *The endothelium of the cornea and its clinical implications 2nd ed* (Charles C Thomas Springfield, 1971)

STOCKER, F W, KING, E H, LUCAS, D O AND GEORGIADE, N *A comparison of two different staining methods for evaluating corneal endothelial viability* Arch Ophthalmol **76:** 833-838, 1966

STOKER, M G P AND RUBIN, H *Microbiology Density dependent inhibition of cell growth in culture* Nature **215:** 171-172, 1967

STONE, D L, KENYON, K R AND STARK, W J *Ultrastructure of keratoconus with healed hydrops* Am J Ophthalmol **82:** 450-458, 1976

SVEDBERGH, B *Effects of artificial intraocular pressure elevation on the corneal endothelium in the Vervet monkey* Acta Ophthalmologica **53:** 839-855, 1975

SVEDBERGH, B AND BILL, A *Scanning electron microscopic studies of the corneal endothelium in man and monkeys* Acta Ophthalmologica **50:** 321-336, 1972

TOST, M, SCHULZE, F AND SHISKO, G *Zur Kalteschädigung des Hornhautendothels* Ophthalmologica **155:** 87-97, 1968

TRIPATHI, R C *Ultrastructure of the normal cornea,* in 'Corneal Grafting' (Edited by Casey, T A , Butterworth & Co Publishers Ltd , London, pp 38-59, 1972)

VAN HORN, D L AND HYNDIUK, R A *Endothelial Wound Repair in Primate Cornea* Exp Eye Res **21:** 113-124, 1975

VAN HORN, D L, SENDELE, D D, SEIDEMAN, S AND BUCO, P J *Regenerative capacity of the corneal endothelium in rabbit and cat* Invest Ophthalmol Vis Sci **16:** 597-613 1977

VON BAHR, G *Corneal thickness its measurment and changes* Am J Ophthalmol **42:** 251-266, 1956

VON SALLMAN, L CARAVAGGIO, I I AND GRIMES P *Studies on the corneal endothelium of the rabbit I Cell division and growth* Am J Ophthalmol **51:** 955-969, 1961

VON SALLMAN, L, GRIMES, P AND MCELVAIN, N *Studies on the corneal endothelium of the rabbit II The generative cycle of the cell* Arch Ophthalmol **69:** 815-823, 1963

WALLER, W K *Die Regeneration des Hornhautendothels nach Kalteanwendung* in 'Wundheilung des Auges und ihre Komplikationen' (Edited by Naumann G O H und Gloor B P , J F Bergmann Verlag, Munchen pp 227 230 1980)

WALLS, G L *The vertebrate eye and its adaptive radiation* Cranbook Ins Sc Bull **19,** 1942

WALTMAN, S R *The cornea,* in 'Adlers' Physiology of the eye, seventh ed ' (Edited by Moses, R A , The C V Mosby Compagny, St Louis, pp 38-62, 1981)

WALTMAN, S R AND KAUFMAN H E *In vivo studies of human corneal endothelial permeability* Am J Ophthalmol **70:** 45-47 1970

WARING, G O, LAIBSON, P R AND RODRIGUES, M *Clinical and pathologic alterations of Descemet's membrane with emphasis on endothelial metaplasia* Surv Ophthalmol **18:** 325-368, 1974

WEIMAR, V L, SQUIRES, F L AND KNOX, R J *Acceleration of healing of rabbit corneal endothelium by mesodermal growth factor* Invest Ophthalmol Vis Sci **19:** 350-361, 1980

WERB, A *Complications of full thickness keratoplasty,* in 'Corneal Grafting' (Edited by Casey, T A , Butterworth & Co Publishers Ltd , London, pp 110-120, 1972)

WICKHAM, M G AND BINDER, P S *Evaluation of corneal endothelial damage using correlated microscopy techniques* Ophthalmic Res **8:** 407-413, 1976

WINTER, F C *The histopathology of unsuccessful penetrating keratoplasty in man,* in 'Cornea Plastic Surgery' (Edited by Rycroft, P V , Pergamon Press, Oxford, pp 557-569, 1969)

WITMER, R *Wundheilung bei perforierendere Keratoplastik,* in 'Wundheilung des Auges und ihre Komplikationen' (Edited by Naumann, G O H und Gloor, B P , J F Bergmann Verlag, Munchen, pp 191-194, 1980)

WOLTER, J R *Detachment of the corneal endothelium In purulent keratitis and ulceration* Am J Ophthalmol **64:** 449-453, 1967

WULLE, K G AND LERCHE, W *Electron Microscopic Observations of the early development of the human corneal endothelium and Descemet's membrane* Ophthalmologica **157:** 451-461, 1969

WULLE, K G, RUPRECHT, K W AND WINDRATH, L C *Electronmicroscopy of the development of the cell junctions in the embryonic and fetal human corneal endothelium* Invest Ophthalmol **13:**923-934, 1974

YANO, M AND TANISHIMA, T *Wound healing in rabbit corneal endothelium* Jpn J Ophthalmol **24:** 297-309, 1980

YAOI, Y *Role of microexudate carpet in cell division* Nature **237:** 283-285 1972

YAOI, Y, ONODA, T AND TAKAHASHI, H *Inhibition of mitosis in suspension culture of chick embryo cells* Nature **237:** 285-286, 1972

YEH, J AND FISHER, H W *A diffusible factor which sustains contact inhibition of replication* J Cell Biology **40:** 382-388, 1969

YUE, B Y J T, BAUM, J L AND SELBERT, J E *Synthesis of glycosaminoglycans by cultures of normal human corneal endothelial and stromal cells* Invest Ophthalmol Vis Sci 17 523-527, 1978

ZAGORSKI, Z *Replication capacity of the regenerating human corneal endothelium in organ culture* in 'Wundheilung des Auges und ihre Komplikationen' (Edited by Naumann G O H und Gloor, B P, J F Bergmann Verlag, Munchen, pp 223-225, 1980)

ZETTERBERG, A AND AUER, G *Proliferative activity and cytochemical properties of nuclear chromatin related to local cell density of epithelial cells* Exptl Cell Res **62:** 262-270, 1970

ZINN, K M *Changes in corneal ultrastructure resulting from early lens removal in the developing chick embryo* Invest Ophthalmol **9:** 165-182, 1970

CURRICULUM VITAE

Willem Frits Treffers was born in De Bilt on 5th July 1944. After obtaining his final secondary school certificate (sciences) at the Christelijk Lyceum in Veenendaal, he enrolled to study medicine at the medical faculty of the University of Groningen, graduating on 15th July 1971.
From 1st September 1971 to 1st September 1975 he specialized in ophthalmology under Prof. Dr J.E.A. van den Heuvel at the Department of Ophthalmology of the St. Radboud Hospital, University of Nijmegen. Since 1st September 1975 he has been on the faculty of the same department (head: Prof. Dr A.F. Deutman).
From 1st September 1980 to 1st September 1981 he was visiting professor at the Bascom Palmer Eye Institute, Department of Ophthalmology, University of Miami School of Medicine, Miami, Florida, USA.

STELLINGEN

I

Nu is aangetoond, dat bij de corneaendotheelwondgenezing proliferatieve eigenschappen wel degelijk een rol spelen, dient de waarde van de endotheelfotografie (specular microscopy) opnieuw beoordeeld te worden

II

De toepassing van locaal toegediende corticosteroiden in de directe postoperatieve fase bij oogheelkundige patienten is discutabel

III

Een ongestoorde wondgenezing heeft een 'preventief' aspect

IV

Een intraoculaire tumor bij een patient bekend met een maligniteit, dient beschouwd te worden als een metastase totdat het tegendeel bewezen is

V

Bij een glasvochtbloeding van een zodanige intensiteit dat funduscopie niet meer mogelijk is, dient op korte termijn echografisch onderzoek verricht te worden met zowel de A als de B scan

VI

De klinische betekenis van fluorometrie is dubieus

VII

De panretinale lichtcoagulatie bij proliferatieve diabetische retinopathie, is een bewezen waardevolle aanwinst bij de behandeling van deze afwijking

VIII

Voor het gebruik van medische stereofotografie is standaardisatie essentieel

IX

De overwaardering van de onderzoekslijn, in het kader van de subsidieverlening, werkt remmend op het aangaan van nieuwe samenwerkingsverbanden tussen onderzoekers

X

Het voorschrijven en het afleveren van medische hulpmiddelen dient gescheiden te blijven.

XI

Hoewel dit doorgaans ontkent wordt, vertoont het gedragspatroon van de mens opvallende gelijkenis met dat van de dieren.

XII

Dat elke generatie opnieuwe gelijksoortige fouten maakt, is grotendeels terug te voeren op het feit dat ervaring helaas nauwelijks overdraagbaar is.

XIII

Door afwezigheid van competitie ontstaat gezapigheid en kwaliteitsverlies.

XIV

Zelfkritiek is één van de gemakkelijkst te onderdrukken karaktereigenschappen.

W.F. Treffers

Mook, 16 september 1982.